Pharmacogenetic significance of inosine triphosphatase

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Inosine triphosphatase (ITPase) is the enzyme that catalyzes the conversion of inosine triphosphate (ITP) and deoxy-inosine triphosphate (dITP) to inosine monophosphate and deoxy-inosine monophosphate, respectively, thereby maintaining low intracellular concentrations of ITP and dITP. Individuals deficient in ITPase activity were first recognized over 30 years ago. For decades, no clinical significance could be attributed to this inborn error of metabolism whatsoever. In recent years, evidence has started to accumulate that polymorphisms in the gene encoding ITPase are associated with potentially severe adverse drug reactions towards the thiopurine drugs azathioprine and 6-mercaptopurine. The pharmacogenetic significance is presently being debated in the literature. However, the present state of knowledge is still insufficient to definitively determine the pharmacogenetic significance of ITPase. This article aims to review the current knowledge on the role of ITPase in thiopurine metabolism.

Keywords: allelic variant, adverse drug reactions, azathioprine, inborn error of metabolism, inosine triphosphatase, inosine triphosphate pyrophosphohydrolase, mercaptopurine, pharmacogenetics, thiopurine, thiopurine methyltransferase

Thiopurines (such as azathioprine, 6-mercaptopurine and 6-thioguanine) are cytotoxic drugs used for the treatment of patients suffering from a diversity of immunological and lymphoproliferative diseases. The most frequently used thiopurine-drugs are azathioprine and 6-mercaptopurine (6-MP). Thiopurines are anti-metabolites, that is, they inhibit physiological processes by replacing their natural counterparts (guanosine nucleotides). Thiopurines must be activated by the purine salvage pathway to exert their cytotoxic effects [1]. The 6-thioguanosine nucleotides inhibit RNA and DNA synthesis, thereby inducing cell death. At least part of the immunosuppressive properties of azathioprine is due to binding of 6-thio-GTP to Rac1 instead of GTP, stimulating the mitochondrial apoptotic pathway in human $CD4^+$ T lymphocytes [2,3].

A well-known cause of potentially severe thiopurine-induced adverse drug reaction (ADR) is decreased or absent activity of the enzyme thiopurine *S*-methyltransferase (TPMT). For a long time, TPMT deficiency was thought to be the only enzyme defect causing ADRs in thiopurine therapy. However, in recent years evidence has been published that polymorphisms in the gene encoding inosine triphosphatase (ITPase) may be associated with the occurrence of ADRs in patients treated with azathioprine [4,5]. This review will discuss the place of ITPase in thiopurine metabolism and its pharmacogenetic significance.

ITPase in purine metabolism

Purines are essential compounds. Together with the pyrimidines, they are the precursors of DNA and RNA. Purines are involved in numerous cellular processes, for example transport and transfer of energy, signal transduction and the biosynthesis of cofactors. Purine nucleotides are synthesized via two routes (**Figure 1**). In proliferating cells, an energetically costly *de novo* synthesis route is used to synthesize the central purine nucleotide inosine monophosphate (IMP) from phosphoribosylpyrophosphate. In resting cells, or cells that do not express the complete *de novo* pathway, IMP is synthesized by reutilization of the purine base hypoxanthine via hypoxanthine guanine phosphoribosyl transferase (HGPRT; EC 2.4.2.8). From IMP, the nucleotides inosine diphosphate (IDP) and inosine triphosphate (ITP) are synthesized by nucleoside monophosphate kinase and nucleoside diphosphate kinase, respectively. Inosine nucleotides other than IMP may be regarded as by-products of purine metabolism. No physiological function has yet been attributed to these metabolites.

In normal cells, no ITP or deoxy-inosine triphosphate (dITP) is found due to efficient pyrophosphohydrolysis of these nucleotides to their respective monophosphates by ITPase (EC 3.6.1.19). Apparently, IMP, IDP and ITP exist in a futile cycle that is sustained by the nucleotide kinases and ITPase. This cycle is crucial for maintaining low intracellular levels of ITP and dITP. Accumulation of ITP may be considered to be undesirable, as it may interfere in several cellular processes, such as GTP-requiring reactions [6]. ITP may also interfere in ATP-requiring reactions, for example ITP has been shown to be a substrate for F_0-F_1-ATPase *in vitro* [7]. Further-

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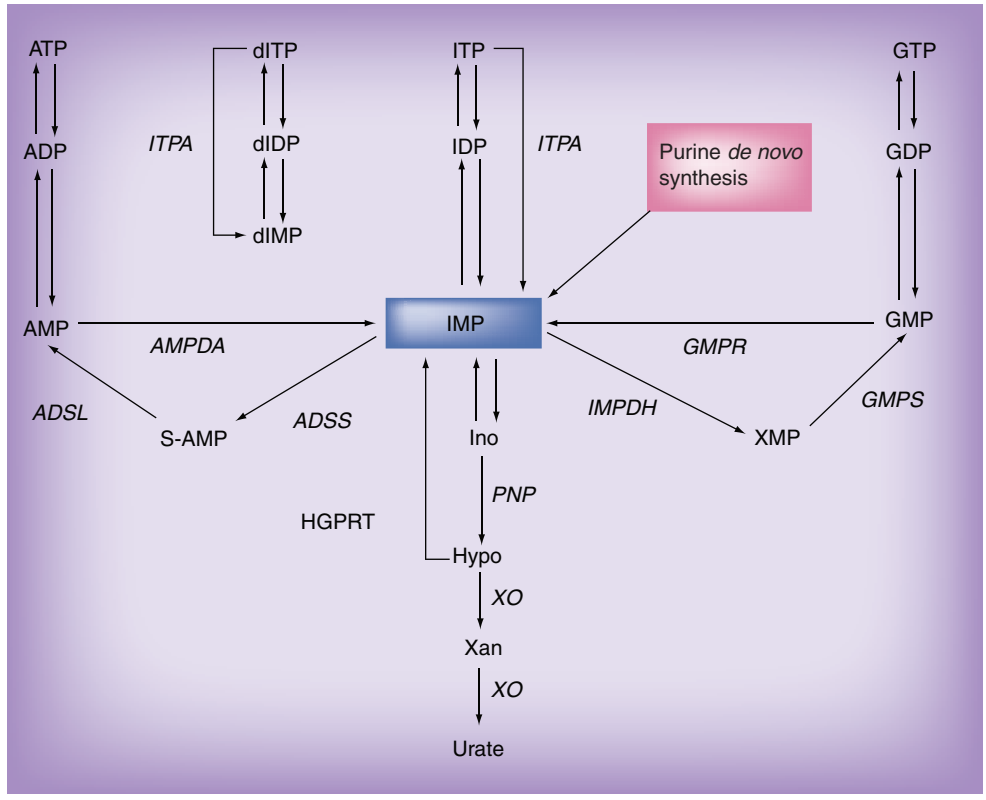
Figure 1. Overview of purine metabolism.



ADSL: Adenylosuccinate lyase; ADSS: Adenylosuccinate synthetase; AMPDA: AMP deaminase; GMPR: Guanosine monophosphate reductase; GMPS: Guanosine monophosphate synthetase; Hypo: Hypoxanthine; HGPRT: Hypoxanthine guanine phosphoribosyltransferase; Ino: Inosine; IMPDH: Inosine monophosphate dehydrogenase; ITPA: Inosine triphosphatase; PNP: Purine nucleoside phosphorylase; S-AMP: Succinyl-adenosine-5′-monophosphate; Xan: Xanthine; XMP: Xanthosine-5′-monophosphate; XO: Xanthine oxidase

more, ITPase deficiency may cause an accumulation of dITP. dITP is incorporated into DNA by DNA polymerases both in cell lysates and intact cell systems [8], thereby rendering DNA-synthesis error prone. In fact, dITP is used in site-directed mutagenesis [9].

Inborn error of metabolism

Deficiency of ITPase (OMIM 147520) is an inborn error of metabolism and was first published over 30 years ago by Vanderheiden [10]. Enzyme activity assays and catalytic properties were published soon thereafter [10–12]. However, it was not until 2002 that the genetic basis of ITPase deficiency was elucidated [6,13]. The crystal structure of ITPase was published [14] and possible molecular mechanisms of ITPase deficiency were proposed [15,16]. There are two likely mechanisms of ITPase deficiency resulting from the *ITPA* c.94C>A mutation. Arenas and colleagues proposed that *ITPA* c.94C>A destroys an exonic splicing-silencing element in exon 2, resulting in the activation of two nearby upstream 5′ splice sites and mis-splicing of the exons 2 and 3 cassette causing structural changes to the enzyme, contributing to ITPase deficiency [15]. Stenmark and colleagues predicted that the P32T amino acid substitution resulting from the *ITPA* c.94C>A mutation causes a structural rearrangement of α-helix 2, possibly leading to a disturbed nucleotide exchange or a partial miss folding of α-helix 2, possibly rendering the dimer inactive [14].

A clinical phenotype was expected to be associated with ITPase deficiency, and studies were performed in patients suffering from mental retardation [17] and schizophrenia [18]. No correlation between ITPase deficiency and a clearly defined clinical presentation could be made. The occurrence of ITPase deficiency in a kindred with adenosine deaminase deficiency proved to be coincidental [19]. The fact that no clear clini-

cal presentation is associated with ITPase deficiency is in contrast with other inborn errors of metabolism directly or indirectly affecting nucleotide metabolism. However, in our opinion, it is, not yet not excluded that ITPase deficiency may be a modulating factor that may aggravate the clinical presentation of other defects in nucleotide or nucleic acid metabolism.

ITPA genetics

The gene encoding ITPase, *ITPA,* is located on chromosome 20p13 [20]. The gene spans 8 exons and has a length of approximately 13 kb [6]. Thus far, five allelic variants have been described that are associated with decreased enzyme activity (**Table 1**). The possible pharmacogenetic consequences of these polymorphisms are discussed below. To date, *ITPA* c.94C>A is the sole allelic variant that may be associated with azathioprine/6-mercaptopurine-induced ADRs. The distribution of *ITPA* c.94C>A has been studied by several groups and was reviewed by Marsh and colleagues [21]. *ITPA* c.94C>A is found in all populations. However, the allele frequency of *ITPA* c.94C>A varies dramatically between populations, being lowest in Central- and South-American populations (1–2%) and highest in Asian populations (11–19%). In Caucasian, African-American and African populations the allele frequency of is 5–7% [21].

The allele frequency of *ITPA* g.IVS2+21 A>C is approximately 13% in Caucasian populations [6,22] and was not found in a Japanese population [23].

ITPA allelic variants & thiopurine-induced ADR

Thiopurine metabolism is an intricate network of enzymatic and nonenzymatic reactions and has been reviewed previously [1,24,25]. The significance of ITPase in thiopurine-based therapeutic regimens is at present a focal point of discussion. In 2004, Marinaki and colleagues published the first reports that polymorphisms in *ITPA* are associated with azathioprine induced ADR [4,5]. In a retrospective study of patients suffering from inflammatory bowel disease (IBD), the occurrence of side-effects such as rash (odds ratio [OR]: 10.3; 95% confidence interval [CI]: 4.7–62.9; p = 0.0213), flu-like symptoms (OR: 4.7; 95% CI: 1.2–18.1; p = 0.0308) and pancreatitis (OR: 6.2; 95% CI: 1.1–32.6; p = 0.0485) was significantly correlated with *ITPA* c.94C>A [4]. In a prospective study by von Ahsen and colleagues, patients carrying *ITPA* c.94C>A were significantly more likely to withdraw early from azathioprine therapy (OR: 11.3; 95% CI: 2.5–50.0; p = 0.001) [26]. Withdrawal at any moment due to azathioprine attributable side effects (myelosuppression, hepatotoxicity, pancreatitis and flu-like symptoms) was also associated with the ITPA c.94C>A carrier status (OR: 7.8; 95% CI: 2.1–29.1; p = 0.002) [26]. In another study, the *ITPA* c.94C>A genotype appeared to predict azathioprine-induced myelosuppression (OR: 3.504; 95% CI: 1.119–10.971; p = 0.046), but not the occurrence of hepatotoxicity [27]. Gearry and van Dieren found no correlation between *ITPA* status and any ADR (OR: 1.015; 95% CI: 0.360–2.867; p = 0.593) in their respective studies [28,29]. However, no conclusion could be drawn for ITPA c.94C>A homozygous individuals [29]. Allorge found no correlation between *ITPA* genotype and azathioprine/6-mercaptopurine induced myelosuppression, but omitted other ADRs [30]. De Ridder and colleagues found no correlation between *ITPA* c.94C>A and ADRs (leucopenia, pancreatitis and 'general malaise') in pediatric patients. Two patients homozygous for *ITPA* c.94C>A tolerated azathioprine well [31]. The study of Breen in which no correlation was found between azathioprine-induced ADR and *ITPA* genotype in patients who received a liver transplant [32] may be due to the fact that the patients' own genotype and the genotype of the liver received do not necessarily correspond. An individual homozygous for *ITPA* c.94C>A receiving a liver with fully functional ITPase may very well be rescued from azathioprine-induced toxicity by the donor liver.

Thus far, only *ITPA* c.94C>A was found to be associated with thiopurine associated ADRs by some authors. Whether or not an allelic variant has pharmacogenetic consequences may be dependent on the residual enzyme activity. *ITPA* c.94C>A heterozygotes have approximately 25% residual activity whereas homozygotes have zero activity [4,26]. However, both heterozygotes and homozygotes for *ITPA* g.IVS2+21A>C, and *ITPA* g.IVS2+68T>C have significant residual activity (≥60%) [4,33]. These genotypes have not been found to be associated with ADRs. *ITPA* g.IVS2+68T>G [23] and *ITPA* 359_366dupTCAGCACC [22] are more likely to have pharmacogenetic significance, as heterozygotes have similar residual activity to ITPA c.94C>A heterozygotes [4,26], and zero residual activity can be expected in homozygotes.

Since ITPase is not involved in the catabolism of 6-thioguanine, the use of 6-thioguanine

Table 1. Allelic variants of *ITPA* associated with decreased enzyme activity.

Genotype	Residual enzyme activity	Association with ADR reported	Ref.
ITPA c.94C>A	Heterozygote: 22.5% Homozygote: <1%	Yes	[4,6]
ITPA g.IVS2+21 A>C	Heterozygote: 60%	No	[4,6]
*ITPA g.*IVS2+68T>G	Heterozygote: 30%	Not yet reported	[23]
ITPA g.IVS2+68T>C	Heterozygote: 60%	No	[33]
ITPA 359_366dupTCAGCACC	Heterozygote: 30%	Not yet reported	[22]

ADR: adverse drug reaction; ITPA: Inosine triphosphatase

instead of azathioprine for individuals with ITPase deficiency would from a biochemical point of view be an alternative. However, in reality 6-thioguanine gives rise to many serious side-effects and is therefore less attractive. When 6-thioguanine is used it is of the utmost importance to be aware of the TPMT status of the patient. One should bear in mind that thiopurine metabolism is complex and is at present far from being fully elucidated.

ITPase substrate specificity & its implications

The fact that ITPase catalyzes the pyrophosphohydrolysis of ITP, dITP and xanthosine-5′-triphosphate is well established [11,12,34]. However, while the assumption that ITPase catalyzes the pyrophosphohydrolysis of the 6-MP metabolite 6-thio-ITP to 6-thio-IMP is generally made, it has never been demonstrated that 6-thio-ITP is actually a substrate for ITPase. We have determined the substrate specificity of human erythrocyte ITPase towards 6-thio-ITP and 6-methylthio-ITP, and compared it to the substrate specificity of ITP. As shown in **Table 2**, ITP is the preferred substrate for human erythrocyte ITPase. 6-thio-ITP is also efficiently converted to 6-thio-IMP, whereas 6-methylthio-ITP was a poor substrate. Michaelis-Menten kinetics were observed for all substrates. Together with the previous observation that RNA polymerase is inhibited by 6-thio-ITP [35] our findings are in support of the possible pharmacogenetic significance of ITPase. We propose the metabolism of 6-MP and azathioprine as depicted in **Figure 2**. A secondary increase in accumulation of 6-methylthio-ITP may occur in cells lacking ITPase activity when challenged with 6-MP. This is supported by the finding that 6-methylthio-ITP, but not 6-thio-ITP is detected in (white blood) cells obtained from bone marrow aspirates and peripheral blood samples [36,37]. This indicates that the 6-thioinosine nucleotides are substrates for TPMT. Further detailed *in vitro* studies of the metabolism of (methyl)thiopurine nucleotides are needed to further elucidate role of ITPase. Preferably, the thiopurine nucleotide distribution should also be studied in peripheral blood cells obtained from individuals treated with 6-MP or azathioprine. Although erythrocytes are not capable of purine *de novo* synthesis, their nucleotide content is thought to reflect the nucleotide status of the liver and other tissues [1].

Whilst high performance liquid chromatography (HPLC) analysis of hydrolyzed thiopurine nucleotides contained within erythrocytes is useful for therapeutic drug monitoring [38,39], it provides no information on the fractions of the nucleotides present. Only by meticulous study of natural purine nucleotides and thiopurine nucleotides in human cells can the pharmacogenetic significance of ITPase be elucidated.

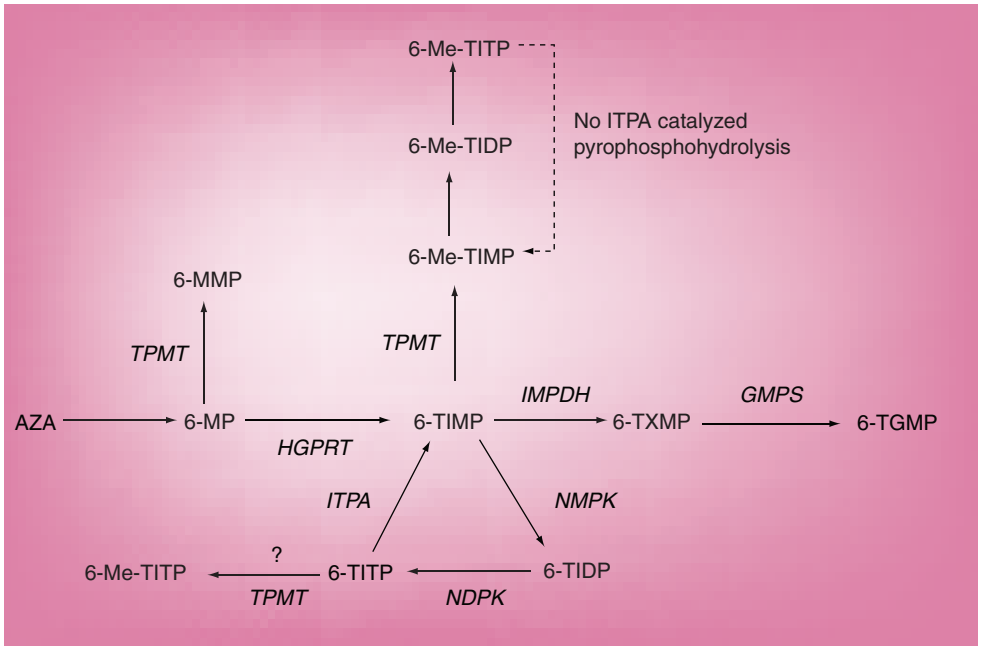
Expert commentary

The results of clinical studies investigating the pharmacogenetic significance of ITPase in thiopurine-based therapy are contradicting. Correct assessment of the pharmacogenetic significance of ITPase is hampered by the small number of patients in each study and the different approaches used. More extensive and preferably

Table 2. Substrate specificity of human erythrocyte ITPase.

Substrate	K_M (μM)	V_{max} (μmol/mg protein/h)	V_{max}/K_M
Inosine-5′-triphosphate	107	1068	9.9
6-thio-inosine-5′-triphosphate	283	512	1.8
6-methylthio-inosine-5′-triphosphate	924	335	0.4

Figure 2. Putative involvement of ITPAse in the metabolism of azathioprine.



6-MP: 6-mercaptopurine; 6-TIMP, 6-TIDP; 6-TITP: 6-thio-inosine-5´-mono-, di and triphosphate; respectively, 6-Me-TIMP; 6-Me-TIDP, 6-Me-TITP: 6-methylthio-inosine-5´-mono-; di and triphosphate, respectively; 6-TXMP: 6-thio-xanthosine-5´-monophosphate, 6-TGMP: 6-thio-guanosine-5´-monophosphate; Aza: Azathioprine; GMPS: Guanosine monophosphate synthetase; HGPRT: hypoxanthine guanine phosphoribosyltransferase, IMPDH: inosine monophosphate dehydrogenase; ITPA: Inosine triphosphatase, NDPK: Nucleoside diphosphate kinase; NMPK: Nucleoside monophosphate kinase; TPMT: Thiopurine *S*-methyl transferase

prospective studies are needed to definitively determine the pharmacogenetic significance of ITPase. At present it is to early to dismiss or fully accept the pharmacogenetic value of ITPase.

Although screening for the most frequent allelic variant for both *ITPA* and *TPMT* accounts for the vast majority of reduced activities, the chance that patients with rare or novel mutations will be missed cannot be ignored. The enzymatic activities of TPMT and ITPase are easily measured and a clear correlation between genotype and enzyme activity exists [4,6,33]. In our laboratory, we therefore offer both ITPase and TPMT enzyme activity assays as one diagnostic package. Based on the initial reports of Marinaki and colleagues [4,5] and our own findings we decided to implement the ITPase activity assay. For the past 2 years, ITPase activity has been offered as an additional service to the physician and the patient. The physician is informed that the patient may have a decreased tolerance for azathioprine and 6-MP when the ITPase activity measured is in the carrier range or deficient. Awareness of the side effects reported in the literature is raised. In all cases of reduced activity, we recommend monitoring of cellular blood count and liver function. Subsequently, molecular diagnostics are used to confirm the results of the enzyme activity assay. If *ITPA* g.IVS2+21A>C is found, it is reported that this genotype has not yet been associated with azathioprine or 6-MP-induced ADR. In case of reduced TPMT activity in combination with reduced or absent ITPase activity, therapeutic drug monitoring is also recommended. In the event that TPMT deficiency is established, the use of thiopurines is strongly discouraged, regardless of the ITPase status.

Future perspective

In the years to come, the pharmacogenetic significance of ITPase in thiopurine-based therapy will become more clear. Based on substantial clinical studies that remain to be published, ITPase as a risk-factor for thiopurine induced ADRs will either be generally accepted or abolished. Furthermore, there is a dire need for more biochemical data.

The interactions between ITPase and the other key enzymes in thiopurine metabolism need to be

extensively studied. It is not unthinkable that certain 'favorable' and 'unfavorable' combinations of activities and/or polymorphisms in the other proteins involved in purine, DNA and RNA metabolism will become clear. The interactions between ITPase and TPMT and ITPase and IMPDH1 will be worthwhile to study as they will determine the balance between (methyl)thiopurine metabolites and the cytotoxic 6-thio-guanine metabolites. The interaction between ITPase and IMPDH1 is particularly interesting, as one patient resistant to azathioprine therapy has been described with a mutation in the IMPDH P3 promoter [40]. Decreased activity of both IPMDH and ITPase is expected to lead to a massive accumulation of 6-thio-ITP, of which the consequences are still unknown.

Pharmacogenetic significance of ITPase may in theory be expected for all therapies based on inosine analogs. One of these analogs is didanosine (2′,3′-dideoxyinosine), an antiretroviral drug. Preliminary results from our laboratory show that didanosine is a substrate for human erythrocyte ITPase. It may be that the true pharmacogenetic significance of ITPase is to be found in antiretroviral therapy.

Executive summary

- The pharmacogenetic significance of inosine triphosphatase is a highly debated subject and data published are contradictory.
- At present the pharmacogenetic significance of ITPase can neither be established nor dismissed.
- *ITPA* is a polymorphic gene.
- The *ITPA* 94>A genotype may be associated with azathioprine induced adverse drug reaction.
- Azathioprine-induced ADRs-associated with ITPase deficiency may be flu-like syndrome, rash, pancreatitis, myelotoxicity and hepatotoxicity.
- In Western and Asian populations the *ITPA* 94>A allele frequency is 5–19%.
- ITPase activity is easily measured in erythrocytes.
- Carriers of *ITPA* allelic variants are easily detected by measurement of ITPase activity.

Appendix
Materials & methods

The substrate specificity of human erythrocyte ITPase towards ITP (Sigma-Aldrich, Zwijndrecht, The Netherlands), 6-thio-inosine-5′-triphosphate and 6-methylthio-inosine-5′-triphosphate (Jena Bioscience, Jena, Germany) was determined in a pooled erythrocyte lysate obtained from multiple anonymous donors. The assay was essentially performed as previously described [41]. Briefly, pyrophosphohydrolase activity was measured in a final volume of 200 µl containing 100 mM Tris-HCl pH 8.5, 50 mM MgCl2, 0.5 mM dithiothreitol and 31–2500 µM ITP, 6-thio-inosine-5′-triphosphate or 6-methylthio-inosine-5′-triphosphate, respectively. The pyrophosphohydrolysis of ITP and 6-thio-inosine-5′-triphosphate was measured using of 0.2 mg of protein, the pyrophosphohydrolysis of 6-methylthio-inosine-5′-triphosphate was measured using of 0.6 mg of protein. The reaction was started by addition of protein to the assay mixture. The reaction was incubated in shaking water bath at 37°C for 30 min. The reaction was terminated by acid precipitation with perchloric acid and incubated for 10 min on ice. The sample was centrifuged and the supernatant was transferred to a clean vial and neutralized using K_2CO_3. The precipitate was removed by centrifugation and the supernatant was diluted twofold in HPLC-elution buffer and centrifuged over a 0.2 µm nylon filter. HPLC analysis was performed using an ion-pair chromatography. The products were detected using a DAD-detector and calibrated using external standards. Apparent K_M and V_{max} values were deduced from Lineweaver-Burk plots.

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