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Measuring deaminated nucleotide surveillance enzyme ITPA activity with an ATP-releasing nucleotide chimera

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ABSTRACT

Nucleotide quality surveillance enzymes play important roles in human health, by detecting damaged molecules in the nucleotide pool and deactivating them before they are incorporated into chromosomal DNA or adversely affect metabolism. In particular, deamination of adenine moiety in (deoxy)nucleoside triphosphates, resulting in formation of (d)ITP, can be deleterious, leading to DNA damage, mutagenesis and other harmful cellular effects. The 21.5 kDa human enzyme that mitigates this damage by conversion of (d)ITP to monophosphate, ITPA, has been proposed as a possible therapeutic and diagnostic target for multiple diseases. Measuring the activity of this enzyme is useful both in basic research and in clinical applications involving this pathway, but current methods are nonselective and are not applicable to measurement of the enzyme from cells or tissues. Here, we describe the design and synthesis of an ITPA-specific chimeric dinucleotide (DIAL) that replaces the pyrophosphate leaving group of the native substrate with adenosine triphosphate, enabling sensitive detection via luciferase luminescence signaling. The probe is shown to function sensitively and selectively to quantify enzyme activity *in vitro*, and can be used to measure the activity of ITPA in bacterial, yeast and human cell lysates.

INTRODUCTION

Damage to DNA bases in the cell has important impact on human health by causing chromosome instability and mutations that lead to cancer and other diseases. Many cellular pathways exist to find and ameliorate this damage. One of the most common forms of damage that occurs in the cell is hydrolytic and nitrosylation-induced deamination of nucleotide bases, especially for cytosine and adenine (1–3). The most frequent of these is cytosine deamination, leading to uracil (4). The purines adenine and guanine are also deaminated at significant levels, yielding the bases inosine and xanthine, respectively (5,6). The deamination of DNA bases occurs both directly in chromosomal DNA, and also in the cellular nucleotide precursors of DNA (the nucleotide pool). The nucleotide pool is the site of the majority of this lesion, both because deamination is considerably slower in duplex DNA with paired bases, and because of the relatively high concentration of free nucleotides in the cell (7,8).

Although inosine nucleotides are normal intermediates in the *de novo* and salvage pathways of nucleotide metabolism (9,10), higher concentrations are undesirable. Deamination of adenine to inosine in DNA is mutagenic, as DNA polymerases preferentially pair cytosine opposite inosine during replication (11–13). When the deaminated nucleoside triphosphates (dITP/ITP) appear in the nucleotide pool, they can then be incorporated into DNA or RNA (14,15). Incorporation of inosine into RNA may lead to deleterious effects by altering RNA structure or stability, or mistranslation (15,16). Incorporation of deoxyinosine into DNA is non-mutagenic *per se* (17), but intermediates of dI repair can lead to genetic damage including strand breaks and chromosome rearrangements (18,19). Because of these dele-

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terious effects, mechanisms for removal of (d)ITP from nucleotide pools are of high importance. Notably, homologous enzymes capable of conversion of (d)ITP to (d)IMP have been identified in all three domains of life (20).

In eukaryotes, the enzyme believed to be chiefly responsible for surveillance of the products of adenine and guanine deamination in the nucleotide pool is Inosine Triphosphate Pyrophosphatase (ITPA) (20–24). ITPA hydrolytically cleaves the triphosphate moieties of inosine, deoxyinosine, xanthosine, deoxyxanthosine and 6-hydroxylaminopurine (HAP) triphosphate, yielding their nucleoside monophosphate derivatives and releasing pyrophosphate (23–26). The monophosphate forms of damaged nucleotides are not substrates for polymerase enzymes, and their incorporation into DNA or RNA is prevented. This hydrolysis also prevents their cross-reactivity with adenosine triphosphate (ATP)-dependent enzymes.

The human *ITPA* gene was first cloned in 2001; (22) it is a 23.6 kDa protein which functions as a dimer and is localized to the cytosol (27–29). Structural studies (30,31) show a β-sheet fold with a major central nucleotide-binding cleft flanked by α-helix lobes. The purine ring of a bound ITP nucleotide is stacked between two aromatic sidechains, likely adding affinity for the substrate. Specificity for deaminated purines appears to arise from specific interactions of protonated basic sidechains with the 6-keto group of the hydrolyzed nucleobase. Of relevance to this study is the fact that the terminal phosphate residue is exposed to solution, allowing the easy exit of pyrophosphate after bond cleavage (31).

The *ITPA* gene is essential to development; animal models deficient in ITPA activity display genetic instability and developmental abnormalities. Genetic knockout ($Itpa^{-/-}$) mice die before birth or shortly thereafter, and show abnormal heart and brain development (32). These ITPA deficient mice develop high concentrations of ITP, at a level 10% of canonical ATP. $Itpa^{-/-}$ mouse embryonic fibroblasts (MEFs) show increased levels of chromosomal abnormalities (33). Studies of human populations have shown that several genetically inherited variants of the enzyme exist (34). Among the most important is the 94C→A mutation in exon 2, which causes a P32T amino acid substitution. This mutation leads to defects in ITPA mRNA splicing (35) and protein misfolding (36) that results in decreased levels of ITPA in certain tissues (27). This P32T mutation exists at a frequency of up to 15% in some populations (34) and leads to a three-quarter reduction in ITPA activity in erythrocytes of heterozygotes, resulting in increased concentrations of ITP in primary cells (37,38).

The ITPA surveillance enzyme is important to human health in multiple ways (20,24). ITPA deficiency is documented to play a role in adverse responses to purine drugs such as mercaptopurines, used in treatment of lupus erythematosus, histiocytosis, and acute lymphoblastic leukemia (39–41). Also significant is the fact that recent studies have shown that ITPA variants with low enzymatic activity are positively associated with lowered hemolytic anemia induced by ribavirin, used in treatment of hepatitis C (42,43). ITPA mutations have been shown to cause encephalopathy in infants (44), and ITPA deficiency has also been linked to anomalies of brain function and psychiatric disorders (37,38). In addition to these pathologies, ITPA expression has been identified as a prognostic marker of survival in renal cell carcinoma (45). Notably, the human enzyme is reported to be overexpressed in several tumor cell lines, suggesting the enzyme as a diagnostic marker for certain cancers (45,46) and ITPA knockdown has been shown elevate apoptosis caused by adenine analog HAP (47) and to trigger apoptosis in breast cancer cell lines (48). To date, no synthetic small-molecule modulators of ITPA activity have been reported. Such compounds could be useful tools to study the role of this enzyme in a number of disease states; the development of a more convenient and specific *in vitro* assay could facilitate the development of such compounds. Moreover, the broad association of the enzyme's activity with patient responses to nucleoside therapies highlights a clinical need for measuring this activity in biological samples.

For these reasons, it would be useful to be able to study activities of ITPA conveniently both with the purified enzyme *in vitro* and in the cellular context as well. The only existing non-chromatographic method for measuring ITPA activity quantifies the pyrophosphate product of the reaction, using a colorimetric assay of inorganic phosphate (28). However, because pyrophosphate is formed from many biochemical reactions, such an assay is not selective for ITPA over many other enzymes. Moreover, it is not suitable for assay of ITPA activity from biological samples because pyrophosphate is ubiquitous in cells, growth media and biological fluids, and is produced continually by many enzymes in the sample (49). Access to a simple luminescence assay that is specific to ITPA would be a significant step in ITPA-related studies. In addition, such an assay could also be biomedically useful, by assisting in searches for new inhibitors of these pathways, by aiding in patient prognosis during antiviral and anticancer chemotherapy and possibly for patient evaluation for future targeted therapeutics. In response to these needs, we describe here the development of an ITPA-specific molecule, DeoxyInosine ATP-Linked probe (DIAL probe) that directly acts as a substrate for the enzyme, and yields readily detectable and quantifiable signals of ITPA activity.

MATERIALS AND METHODS

General

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 400 MHz NMR spectrometer. ^{1}H and ^{31}P NMR spectra were internally referenced to the residual solvent signal. Semi-preparative high performance liquid chromatography was performed on a LC 20AD Shimadzu liquid chromatography system, equipped with a SPD-M20A diode array detector and a CBM-20A system controller and using reverse phase (C18) columns. Absorbance spectra were obtained on a Cary 100 Bio UV-Vis spectrometer. The bioluminescence signal was recorded by microplate fluorometer (Fluoroskan Ascent, Thermal).

Chemicals were purchased from commercial suppliers (Sigma-Aldrich or Alfa Aesar) unless otherwise indicated. Recombinant human inosine triphosphate pyrophosphatase protein was purchased from Abcam (Cambridge, USA). The Kinase-Glo™ Luminescent Kinase Assay kit

(Promega) was used for the bioluminescence assay. A Protein Assay Kit (Bio-Rad) was used for determination of the protein concentration of cell lysates.

Synthesis of deoxyinosine monophosphate-ATP chimera

The sodium salts of dIMP and ATP were separately dissolved in distilled deionized water and converted into their free acid forms using a Dowex-50W ion exchange column (H^+ form), titrated to pH 7.0 with a dilute solution of tetrabutylammonium hydroxide, and then lyophilized twice to powder. The lyophilized powders of the nucleotides were coevaporated with anhydrous Dimethylformamide (DMF) twice and stored under high vacuum for 3 h before the subsequent coupling reaction. For the coupling step, the tetrabutylammonium salt of ATP (80 mg, 50 μmol) was dissolved in 1 ml anhydrous DMF. To this solution, carbonyldiimidazole (CDI, 50 mg, 300 μmol) was added, and the mixture was stirred at room temperature for 5 h, after which 50 μl MeOH was added to quench the reaction. All solvents were removed under high vacuum and the residue redissolved in 1 ml anhydrous DMF. The 2′-Deoxyinosine monophosphate tetrabutylammonium salt (30 mg, 30 μmol) in 1 ml DMF and anhydrous $MgCl_2$ (10 mg) was added. The mixture was stirred 72 h at room temperature. After this, the product was precipitated by the addition of acetone (10 ml). The precipitate was washed twice with 10 ml acetone. The desired product was purified by reverse-phase HPLC using a preparative C18 column with a gradient of acetonitrile and 50 mM triethylammonium acetate buffer (pH 7). Fractions containing pure product were concentrated and further purified by a DEAE Sephadex G-25 anion exchange column, eluting with 500 mM NH_4HCO_3. The fractions containing the desired product were pooled, concentrated and repeatedly freeze-dried to yield the final product as a white powder in a yield of 55%. ^{1}H NMR (D_2O, 400 MHz, NH_4^+ form): δ 8.25 (s, 1H), 8.12 (s, 1H), 7.96 (s, 1H), 7.92 (s, 1H), 6.19–6.15 (m, 1H), 5.86–5.85 (m, 1H), 4.54–4.52 (m, 2H), 4.37–4.36 (m, 1H), 4.15–4.14 (m, 1H), 4.12–3.99 (m, 5H), 2.56–2.52 (m, 1H), 2.39–2.36 (m, 1H). ^{31}P NMR (D_2O, 162 MHz, NH_4^+ form): δ -10.17, -10.17, -21.94, -22.02. HRMS: calculated for $C_{20}H_{27}N_9O_{19}P_4$ $(M - H)^-$ 820.0301; found 820.0275.

See Supplementary Data for further characterization data.

Enzyme and luciferase methods

A standard 20 μl reaction buffer contained: 50 mM Tris–HCl, pH 8.5, 50 mM $MgCl_2$, 1 mM Dithiothreitol (DTT), 100 μM DIAL nucleotide, and varied amounts of ITPA. After 1 h incubation at 37°C, 5 μl of this reaction solution was added to 20 μl Kinase-Glo Luminescent Kinase Assay reaction solution (prepared as instructed for ATP determination kit) in a 96-well plate. The bioluminescence signal was recorded at 1 min intervals over 1 h by microplate fluorometer.

Enzyme kinetics and inhibition

A substrate inhibition curve was generated by adding ITPA (20 nM) in 50 mM Tris–HCl, pH 8.5, 50 mM $MgCl_2$,1 mM DTT and various amount of DIAL (16–1000 μM) at 37 °C for 10 min. Then 5 μl of the reaction solution was added to 20 μl Kinase-Glo Luminescent Kinase Assay reaction solution to calculate the amount of ATP generated by ITPA reaction. All the reactions were performed in three replicates. The velocity was plotted as a function of DIAL concentration and fit with the substrate inhibition equation ($Y = V_{max}*X/(K_m + X*(1 + X/K_i))$) to obtain the kinetic parameters, V_{max} and K_m. The k_{cat} values were calculated by dividing the V_{max} by the concentration of ITPA used.

Cell lysates

Bacteria. *Escherichia coli* strains AB1157 (wild-type) and ITPA deficient JB29 (*rdgB62*) described in reference (50) were kindly provided by Dr Kuzminov (University of Illinois, Urbana-Champaign). The strains are isogenic and have additional genetic markers: F- lambda- rac- *thi-1 hisG4Δ (gpt-proA)62 argE3 thr-1 leuB6 kdgK51 rfbD1 araC14 lacY1 galK2 xylA5 mtl-1 tsx-33 glnV44 rpsL31)*. *Escherichia coli* overnight cultures were diluted 100-fold by fresh LB broth and grown up to OD 600 1.5–3. Then bacterial cells were pelleted by centrifugation and resuspended in 5–10× volumes of the bacterial lysis buffer (50 mM Tris pH 8.5, 50 mM NaCl, 1.5 mM $MgCl^2$, 1 mM DTT, protease inhibitor cocktail (Promega # G6521) and 0.2 mg/ml lysozyme). The cell suspension was gently pipetted up and down until homogeneous and incubated at 37°C for 30 min. At the end of the incubation period, the tubes were inverted several times to complete the lysis. Then crude lysates were quickly frozen and kept at −80°C for 3 min and then thawed quickly in 42°C water bath and vortexed vigorously. The freeze/thaw cycle was repeated four times. Lysis was further assisted by pipetting the suspension up and down a few times with a narrow bore pipet tip or a 20-gauge syringe needle. Cell lysates then were treated with RNAse A at 37°C for 60 min. After the treatment cell lysates were centrifuged at 20 000 × *g* for 30 min at 4°C and the clear lysate was collected.

Yeast. To study ITPA activity in yeast cell extracts we constructed two strains: wild-type strain ES-15 and ITPA deficient strain ES-18 (*ham1*). ES-15 strain was obtained on the base of the strain Δ1, a psi-minus derivative of Δl(-2)l-7B-YUNI300 (51) *(MATa ade2–1 his7–2 leu2Δ::kanMX ura3Δ trp1–282 lys2ΔGG2899–2900 psi-)* by conversion of the mutant allele *ade2–1* to the wild-type *ADE2*. First we amplified the *ADE2* gene using genomic DNA of the BY4742 strain as a template and two primers ADE2-F CGGACA AAACAATCAAGTATGG and ADE2-R TTGATGTA ATCATAACAAAGCC. Then 1818-bp PCR product was used to transform the Δ1 strain. Transformants were selected for adenine prototrophy. ES-18 strain, ITPA deficient derivative of the ES-15, was obtained by disruption of the *HAM1* gene with the *LEU2* as described before (52). Both ES-15 and ES-18 strains where cultured overnight in 20–25 ml of complete YPDAU media (standard YPD supplemented with adenine and uracil). Then cells were collected by centrifugation. Equal volumes (aprox. 200 μl) of cell disruption buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1 mM DTT, protease inhibitor cocktail (Promega) and

0.5 ml glass beads were added to each tube. To disrupt cells, tubes were vortexed in a cold room for five intervals (1 min each) with cooling on ice between vortex cycles. Then samples were centrifuged at +4°C for 5 min, 1000 rpm. Supernatant was placed into new tubes and centrifuged one additional time at +4°C for 10 min at 8000 rpm. Clear yeast cell lysates were collected.

Human cells. We used common cancer cell lines HeLa and U2OS. Cells were cultivated in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) + 10% FBS (Gibco) and 1% Penicillin Streptomycin in a humidified incubator with 5% CO_2. The shRNA plasmids against ITPA were purchased from SABiosciences. The plasmids were used to generate stable lines with decreased levels of ITPA by transfection with 10 μg of plasmids (shRNA1 and shRNA2 targeting ORF of ITPA, non-targeting control) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were selected by Puromycin (1 μg/ml). After a week of selection, the surviving cell pools were expanded and RNA was extracted for estimation of knockdown by semi-quantitative RT-PCR. RNA was extracted using the RNA-Zol RNA miniprep kit from ZYMO Sciences or by TRIZOL (Invitrogen), according to the manufacturer's instructions. Single-stranded cDNA was synthesized using the Reverse Transcription System (Promega) and was used as the template for PCR. *ITPA* and *ACTB* encoding for human β-actin were amplified using the following primers using GoTaq from Promega: ITPA Primers F- TCATTGGTGGGGAAGAAGA, R- AAGC TGCCAAACTGCCAAA; primers for human β-actin were as following: forward 5′-ATTGGCAATGAGCGGTTCC G-3′, reverse 5′-AGGGCAGTGATCTCCTTCTG-3′. The products were analyzed by electrophoresis on a 1% agarose gel and imaged using a Kodak Image Station 4000R.

Total protein of HeLa and U2OS cells was extracted using a hypotonic buffer that contained 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 × protease inhibitor cocktail and 1 mM Na_3VO_4 as polymerase inhibitor.

ITPA activity in cell lysates

Once initial lysates are isolated, it is essential to deplete them of ATP prior to DIAL measurement. This depletion was carried out by ATP by centrifugation through an Amicon 3K centrifugal filter (EMD Millipore) as described (53). *Escherichia coli*, *Saccharomyces cerevisiae* and human cells extracts were used to measure ITPA activity after The cell lysates were aliquoted and kept at −80°C until use. Protein concentrations in the cell lysates were measured by Bio-Rad Protein Assay Kit (Bradford method).

All reactions were performed in twelve replicates in ITPA reaction buffer (50 mM Tris–HCl, pH 8.5, 50 mM $MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4). Each reaction of 10 μl volume contained cell lysate with final concentration of total protein 0.5 μg for bacterial and yeast lysates and 1 μg for human cells and 100 μM (200 μM for human lysates) of DIAL nucleotide. Reactions were kept at 37°C (*E. coli* and human cell extracts) or at 30°C (yeast cell extracts) for 15–30 min. Controls with substrate or cell lysate only were also used. After reaction completion 5 μl of the reaction mix was added to 95 μl luciferase reaction solution (ATP Determination Kit, Invitrogen), and the luminescence signal was recorded. In parallel we used ATP to make a standard curve with increasing concentration, up to 1 μM, of ATP. Since DIAL nucleotide probe is contaminated with small amounts of ATP we determined the amount of additional ATP that arose in the presence of cell lysate.

Statistics

Unpaired t-test was used to evaluate the differences in ITPA mRNA levels in human cells. A value of $P < 0.05$ was considered statistically significant (54).

RESULTS

Design of the ATP-linked probe

Our design of an ITPA-specific probe (Figure 1) began with the native deoxyinosine triphosphate structure. ITPA cleaves this native substrate to release pyrophosphate; our concept was to modify this pyrophosphate with an additional phosphate and 5′-linked adenosine. Deoxyinosine was chosen in the design rather than inosine to minimize any chance of cross reactivity with luciferase, which prefers a ribonucleotide substrate. The overall design results in a chimeric ribo/deoxy dinucleotide linked by a tetraphosphate group, termed the DeoxyInosine ATP-Linked (DIAL) probe. In principle, hydrolytic enzymatic cleavage between the phosphates alpha and beta to dI in the DIAL probe should result in expulsion of ATP in place of pyrophosphate during the reaction. Examination of the crystal structure of the enzyme with ITP bound suggests that the terminal phosphate is sufficiently exposed to solution to allow the addition of AMP without severe steric problems in the monomer enzyme (30,31). Recent studies in our laboratory have used a similar ATP-linked chimeric design in development of probes for DNA polymerases and for MTH1, an enzyme that recognizes and cleaves 8-oxodGTP in the nucleotide pool (53). Since ATP can be sensitively and conveniently detected by luciferase and luciferin, this suggests the possibility of direct and specific detection of ITPA activity using commercial luciferase reporter kits. Although the design is straightforward, at the outset it was unknown whether the added bulk of the appended phosphate and adenosine groups might adversely affect enzyme activity, especially in the dimeric form of the enzymes, and whether the DIAL would be selective for ITPA over other enzymes (such as dUTPase and MTH1) (55,56) that process deaminated or oxidized nucleoside triphosphates.

Synthesis of DIAL substrate

The DIAL probe was synthesized by coupling of the commercial available ATP and dIMP (see 'Materials and Methods' section). The sodium salt of ATP and dIMP were transferred to tetrabutylammonium salt first to increase their solubility in organic solvent. ATP was activated with carbonyl diimidazole and coupled with dIMP at room temperature

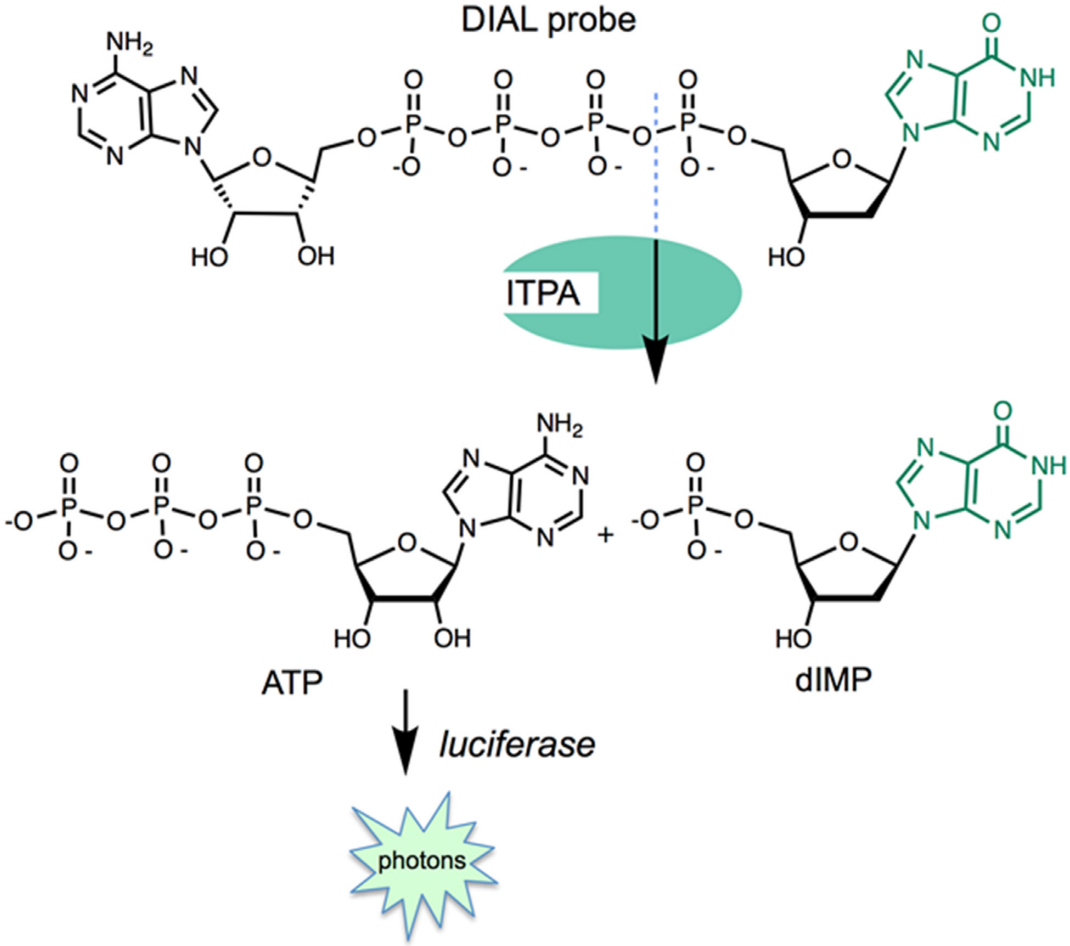


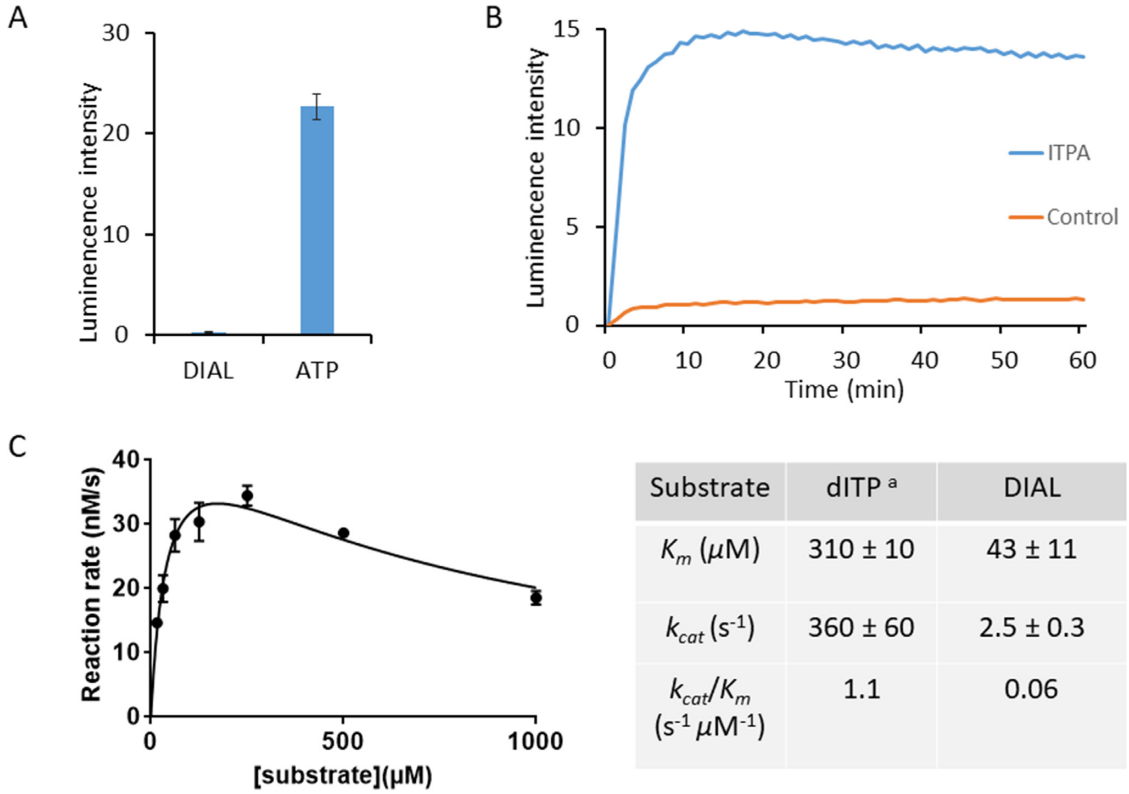
Figure 1. Design of DeoxyInosine ATP-Linked (DIAL) probe. (Top) Structure of DIAL chimeric dinucleotide. (Below) ITPA-mediate cleavage of the probe releases ATP, which is quantified by firefly luciferase-generated luminescence.

for 72 h. The resulting dinucleotide was purified by reverse phase HPLC and then applied to a DEAE column. The final dinucleotide was in ammonium salt and the yield was as high as 55%.

Activity of the probe with purified ITPA

First, we tested the background signal by incubating the DIAL probe with luciferase and monitoring signal as compared with native ATP. The results showed only very small signals for the DIAL dinucleotide compared with ATP. Since the background signals appear rapidly, the signals likely arise from small amounts of contaminating ATP in the synthetic nucleotide, rather than from a slow reaction of DIAL with luciferase. Next, we tested the responses of the probe to ITPA, employing firefly luciferase to measure the generation of ATP. We performed a 50 μl ITPA reaction with 100 μM DIAL, reacted for 2 h and then taking 5 μl aliquots and adding the aliquots to a Kinase Glo™ firefly luciferase reaction (Figure 2). The ITPA enzyme is inactive in the luciferase buffer (data not shown), thus the addition to the second buffer serves as a 'stop' to the reaction. The ITPA reaction buffer generated stable luciferase signals in this commercial luciferase buffer over 10 min, while the control reaction lacking ITPA enzyme (DIAL probe alone) generated extremely small luciferase signals. The experiments establish that DIAL cannot directly act as an efficient luciferase substrate, and requires ATP to be generated by reaction of the DIAL probe with ITPA. A plot of the data (Figure 2B) reveals signals rising over background within 1 min and reaching a plateau at 10 min of luciferase reaction.

We performed kinetics studies of the DIAL substrate with ITPA. The reactions were carried out using 20 nM ITPA and varied concentrations of DIAL. After 10 min reaction at 37°C, the moles of generated ATP were calculated by the luciferase reaction kit, using a standard calibration curve with ATP alone (see Supplementary Data). The experiments reveal that the DIAL probe acts as an ITPA substrate with efficiency significantly lower than native dITP, with K_m values of 43 ± 11 μM, within the range of the reported values of the native substrate (20). The k_{cat} of 2.5 ± 0.03 s^{-1} is significantly lower than the native dITP by a factor of 160-fold. Nevertheless, this level of substrate efficiency was sufficient to allow quantitative measurements of cellular ITPA at constitutive levels (see below). The concentration vs. rate data also reveal that DIAL shows substrate inhibition properties at higher concentrations (Figure 2C). The same is reported to be true for ITP substrate (57,58).



Substrate	dITP [a]	DIAL
K_m (μM)	310 ± 10	43 ± 11
k_{cat} (s^{-1})	360 ± 60	2.5 ± 0.3
k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	1.1	0.06

Figure 2. Substrate properties of DIAL for the ITPA enzyme and firefly luciferase. (**A**) DIAL is a poor luciferase substrate; comparison shows luciferase signals generated by DIAL and by ATP at equal concentrations (100 μM). (**B**) Time course showing generation of luciferase signals (kinase GLO™ assay) subsequent to DIAL/ITPA reaction. Control omits ITPA enzyme. (**C**) Substrate properties of DIAL for ITPA. [a]Published values for ITPA (22).

Sensitivity and selectivity of the probe

We evaluated the sensitivity of the DIAL reporter by varying ITPA concentration and measuring subsequent luciferase signals. A plot of the data (Figure 3) shows a limit of detection of 1 nM enzyme concentration. Later experiments (see below) confirmed that this sensitivity is sufficient to measure levels of ITPA activity in bacterial, yeast and human cell lysates.

Testing the DIAL probe in cell lysates

We examined how ITPA activity could be detected with the DIAL probe in cell lysates of paired samples of wild-type and mutant strains of bacteria *E. coli*, yeast *S. cerevisiae* completely lacking activity of corresponding ITPA gene orthologs (*rdgB* in *E. coli* and *HAM1* in *S. cerevisiae*) and human cells with partially silenced ITPA gene by SureSilencing™ shRNA Plasmids. We have shown that wild-type cells of all tested organisms have robust, though varying ability to cleave the DIAL probe (Table 1 and Figure 4). Highest activity was detected in lysates of *E. coli* cells. In bacteria, activity is 12-fold higher than in yeast extracts after 15 min and 6-fold higher after 30 min of incubation with the DIAL substrate. During longer incubation, the difference between *E. coli* and *S. cerevisiae* lysates decreases, due to a slow-developing background signal that competes with the ITPA signal at later times. In bacterial lysates, level of DIAL hydrolysis reaches its plateau much faster than in yeast extracts. It appeared that all activity in bacteria can be attributed to the function of its ITPA ortholog, because in *rdgB*$^-$ strain no activity was detected (Table 1). Yeast extracts from strain with deletion of the ITPA ortholog, *HAM1* gene, possessed some DIAL breakdown activity seen at longer incubation times (Supplementary Figure S1), suggesting that other enzymes might be able to cleave the DIAL substrate with release of the ATP, albeit at lower efficiency than Ham1.

We tested next how the DIAL probe detects decreased activity of ITPA in extracts of human cells. We engineered cancer cell lines with approximately half of the *ITPA* mRNA (Figure 4A). Even this modest change led to lowered hydrolysis of DIAL probe (Figure 4B), establishing that the DIAL probe can detect ITPA activity in mammalian cells and consistent with the notion that ITPA is one of the main guards against ITP in human cells.

DISCUSSION

We have described a new probe design for ITPA activity, in which the direct product of the reaction, ATP, is directly and sensitively detectable using luciferase, including that in commercial kits. The chimeric DIAL dinucleotide has a simple design, linking ATP and dIMP directly through a tetraphosphate linkage. The compound is conveniently prepared from starting materials in only one step, resulting in good yields. We have shown that the detection of enzymatic activity can be done either in real time (with luciferase present along with ITPA), or in two-step mode, measuring

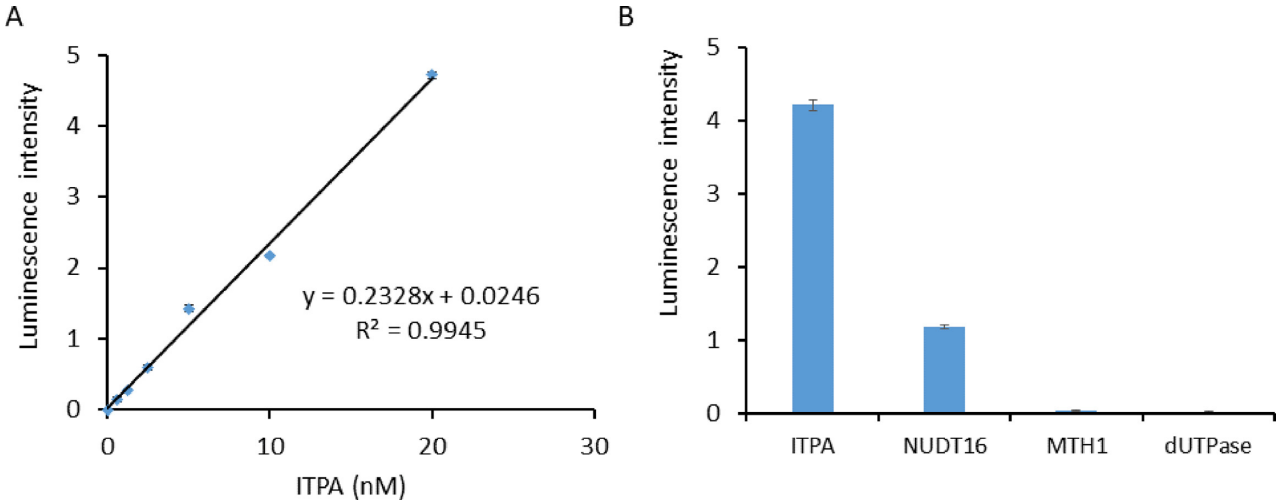


Figure 3. Sensitivity and selectivity of the DIAL probe assay. (**A**) Detection limit of ITPA and (**B**) the selectivity of DIAL probe among four nucleotide surveillance enzymes.

Table 1. Amount of ATP formed in yeast and bacterial cell lysates after varied reaction times

Cells	Strain	ATP formed, nM[§]	
		15 min	30 min
S. cerevisiae	Wild-type	104.6 ± 15.3*	221.3 ± 23.2*
	ham1	30.8 ± 15.9	61.4 ± 17.9
E. coli	Wild-type	1206.3 ± 92.3	1360.5 ± 62.3
	rdgB	Not detected	Not detected

[§]95% confidence interval (CI) for the mean
*The difference between wild-type and ITPA deficient strain at the same reaction time is statistically significant ($P < 0.01$)

luciferase signals at a later time of convenience. This flexibility is potentially important in the design of high-throughput assays.

Performance of the DIAL probe approaches that of the natural substrate in some parameters. The K_m value is within the range of that reported for the native substrate (20,22,28). K_m values in the literature for the enzyme have varied widely from 33 to 600 μM, which may reflect the different methods used to measure it (20). The k_{cat} value we have measured for the DIAL substrate, 2.5 s^{-1}, is considerably lower than that of ITP, which reportedly falls into the range of 79 to 580 s^{-1} (20). While this limits the rate of signal (ATP) generation, this does not appear to adversely affect the utility of the probe, which yields measurable signals over background in conveniently short periods of time. The sensitivity of the DIAL probe shows that it can detect as little as 1 nM of enzyme. This level of sensitivity allows it to clearly detect activities over background in lysates of multiple cell types. Moreover, it is expected to enable its convenient use in high-throughput screens of small-molecule inhibitors of ITPA. At 4 μl per well in 1536-well plates, we calculate that it would be possible to detect ITPA activity in 1536 measurements using only 300 ng enzyme.

The most common assay used in the literature for measuring ITPA activity has been a pyrophosphate-coupled assay, which uses inorganic pyrophosphatase enzyme to breakdown the pyrophosphate product to phosphate, which is subsequently detected colorimetrically (23,57). One of these assays have come under scrutiny because of the question of whether the pyrophosphatase enzyme is limiting in the coupled kinetics (20); moreover, the pyrophosphatase is known to be inhibited by ITP, thus likely interfering with kinetics measurements (57,58). Other assays of ITPA include the use of radiolabeled ITP followed by paper electrophoretic analysis of the IMP product (59), which is relatively slow and is not useful for high-throughput use. A simple HPLC assay, measuring ITP conversion to IMP[32] is perhaps the most common method employed in ITPA activity measurements, has the advantage of direct observation of the native substrate and product in the reaction. However, the method is considerably more time-consuming than the DIAL assay, and (while not tested here) is likely to have lower sensitivity as well, since HPLC detection of IMP is limited by the relatively low molar absorptivity of hypoxanthine. On the other hand, instrumental sensitivity to luminescence (as measured in the DIAL assay) is quite high, and background is extremely low. Thus, in contrast to prior methods, the current DIAL probe assay is faster, simpler, and can function with commercial luciferase kits. We have used a related chimeric dinucleotide assay successfully to screen for inhibitors in high throughput (53), which suggests that the DIAL assay would also be readily applied in such a format.

Our data show that the DIAL substrate is versatile, functioning well with extracts of multiple cell types and ITPA homologs from bacteria, yeast, and human cells. By performing ATP depletion via gel filtration spin columns prior

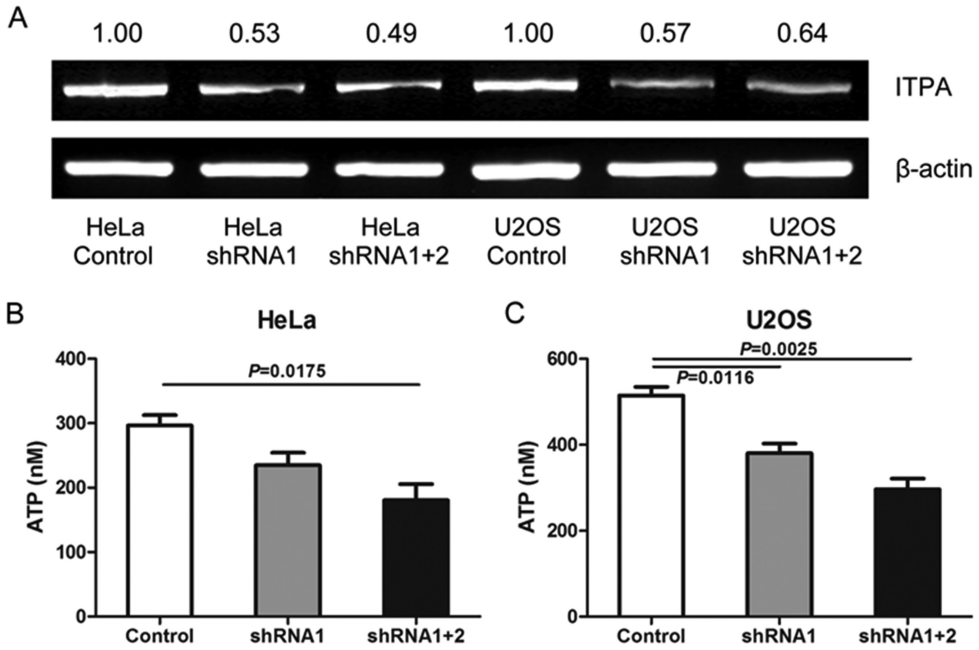


Figure 4. Decrease of DIAL hydrolysis in extracts of human cancer cell lines with lowered levels of *mITPA*. (**A**) Knockdown efficiency by ITPA shRNA in HeLa and U2OS cells analyzed by RT-PCR analysis. The brightness of bands to assess the ITPA expression level was measured by ImageJ. (**B** and **C**) ATP generated in 30 min by extracts of original and ITPA knockdown cell lines.

to measurement, we are able to specifically measure ITPA activity with the selective DIAL substrate. While direct HPLC measurement of cleavage of ITP can be used with lysates of cells or tissues (33,37), it is a relatively slow process. As a result, ITPA expression is typically measured as a proxy for enzymatic activity, using RNA quantitation via quantitative RT-PCR (47). In addition, an antibody-based assay of protein quantity applicable to flow cytometry has been reported (60). However, RNA expression may not correlate with ITPA enzymatic activity due to differences in translation in different cell types, and protein quantity may not correlate well with activity given that polymorphisms in the ITPA gene are known to affect enzymatic activity. For example, the clinically important P32T mutant, found at a frequency of up to 15% in human populations, leads to overall loss of ITPA activity in certain tissues and cell types, leading to significant health issues (39–44). Thus the ability to measure ITPA activity directly will be helpful in the monitoring the health status of P32T carriers.

The ability to rapidly and sensitivity measure ITPA activity in cells and tissues is expected to be broadly useful for a number of applications. These include the basic study of the biology of hydrolytic nucleobase damage and enzymes that recognize this damage, and evaluating the connections between ITPA activity and patient responses to nucleoside therapeutic agents. In addition, a convenient and selective assay of ITPA activity may be useful clinically in predicting which patients are likely to respond to chemotherapy, and in diagnosing any resistance to therapy that arises over courses of treatment (42). Finally, if therapies targeted to ITPA are undertaken (23), it will be necessary to measure the activity of this enzyme both to evaluate patient response and to select appropriate patient populations for treatment. Notably, an analogous chimeric nucleotide assay for a different NUDIX enzyme (MTH1) has been shown to function successfully in primary tumor lysates (53). Although we have not yet confirmed the ability of the DIAL probe to measure ITPA activity in primary blood or tissue samples, the current results measuring activity in human cell lysates demonstrate promise in this direction.

Finally, our studies employing the DIAL probe with yeast cells lacking ITPA gene show that at longer reaction times, cell lysates retain activity in cleaving the DIAL substrate at its alpha-position, exactly analogous to ITPA. This strongly suggests the possibility that in eukaryotes there exist other enzymes with activity overlapping that of ITPA. For example, studies of Itpa$^{-/-}$ MEFs show that they overexpress NUDT16, which has been suggested to compensate for loss of ITPA (33). The primary activity of NUDT16 appears to be hydrolysis of IDP to IMP, but *in vitro* it is reported to have minor activity in hydrolyzing ITP (61). It is possible that the DIAL probe may be a substrate for some minor activity, particularly at long reaction times as seen here. Several other genes, in addition to *HAM1,* are involved in protection from HAP in yeast (62). Future characterization of background activity in extracts of yeast double mutants with deletion of ITPA and these genes or genes encoding other phosphatases will shed light on additional enzymes that participate in cleansing the nucleotide pool of deamination damage in eukaryotes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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