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ATP-Linked Chimeric Nucleotide as a Specific Luminescence Reporter of Deoxyuridine Triphosphatase

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Abstract

Nucleotide surveillance enzymes play important roles in human health, by monitoring damaged monomers in the nucleotide pool and deactivating them before they are incorporated into chromosomal DNA or disrupt nucleotide metabolism. In particular, deamination of cytosine, leading to uracil in DNA and in the nucleotide pool, can be deleterious, causing DNA damage. The enzyme deoxyuridine triphosphatase (dUTPase) is currently under study as a therapeutic and prognostic target for cancer. Measuring the activity of this enzyme is important both in basic research and in clinical applications involving this pathway, but current methods are nonselective, detecting pyrophosphate, which is produced by many enzymes. Here we describe the design and synthesis of a dUTPase enzyme-specific chimeric dinucleotide (DUAL) that replaces the pyrophosphate leaving group of the native substrate with ATP, enabling sensitive detection via luciferase luminescence signaling. The DUAL probe functions sensitively and selectively to quantify enzyme activities *in vitro* and in cell lysates. We further report the first measurements of dUTPase activities in eight different cell lines, which are found to vary by a factor of 7-fold. We expect that the new probe can be of considerable utility in research involving this clinically significant enzyme.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

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NMR spectra of DUAL (PDF)

INTRODUCTION

One of the most common forms of damage in DNA arises from hydrolysis of cytosine, leading to uracil.¹ Uracil in DNA arises both directly, from hydrolytic or enzymatic deamination, and indirectly, from incorporation of the deaminated nucleotide, deoxyuridine triphosphate (dUTP).^{2,3} Deamination of deoxycytidine triphosphate (dCTP) to dUTP in the nucleotide pool is important to the genetic stability of DNA. dUTP is incorporated by polymerases into genomic DNA,^{4,5} and because uracil in DNA is subject to repair pathways, this can lead to double strand breaks and genotoxicity.^{6,7} To prevent problems that arise from deamination of nucleotides in the cellular nucleotide pool, multiple enzymes exist that inactivate these damaged nucleotides for DNA polymerase incorporation.^{8,9}

The enzyme deoxyuridine triphosphatase (dUTPase) is the sole enzyme that cleanses the nucleotide pool of deaminated dCTP.⁸ Homologues of the protein are found in all domains of life. The enzyme recognizes deoxyuridine in dUTP and cleaves the triphosphate moiety between the alpha and beta phosphates,¹⁰ resulting in pyrophosphate and dUMP products, the latter of which is inactivated for DNA synthesis. The human enzyme forms a barrel-shaped homotrimer,¹¹ with an active site aspartate residue (Asp102) that directs water attack at the alpha phosphate of the substrate, expelling pyrophosphate as a byproduct.¹² Nuclear and mitochondrial isoforms of the enzyme exist, both encoded by the same DUT gene and resulting from two splice variants.^{13,14} The nuclear isoform can be phosphorylated at a serine residue by cyclin-dependent kinase and has activity that varies under cell cycle control.¹⁵

Human dUTPase is important to human health in multiple ways.¹⁶ It is under investigation as a prognostic marker for hepatocellular cancer, as high expression levels correlate with poor prognosis in patients with the disease.¹⁷ The dUTPase enzyme is responsible for conferring cellular resistance to fluoropyrimidine antimetabolite drugs commonly used to treat colorectal, breast, and lung cancers.^{18–20} As a result, it is an active therapeutic target,^{21–23} and a dUTPase inhibitor has entered clinical trials as a sensitizer for fluoropyrimidine

drugs.²⁴ In addition to targeting the human enzyme, viral and trypanosomal dUTPase enzymes are also under investigation as possible therapeutic targets as well.^{25,26}

As a result, it is important to be able to quantify dUTPase activities conveniently. Assays that are amenable to high-throughput screening could aid in developing new leads for therapeutics. In addition, measurement of dUTPase activity may well be important in cells and tissues, both for disease prognosis,¹⁷ and potentially to select patients for dUTPase-targeted therapy. However, current methods are indirect, nonspecific, and/or have low sensitivity. Cellular studies commonly measure RNA expression by qRT-PCR or assess protein levels by immunohistochemistry (IHC).²⁰ Those methods are indirect, as they do not measure actual enzymatic activity: RNA quantities may not reflect variable translation levels. Moreover, as there are multiple isoforms of the enzyme^{13,14} and kinase-directed phosphorylation that control levels of activity,¹⁵ IHC-based methods may fail to accurately reflect activity in tissue. *In vitro* methods for measuring the enzyme's activity are based either on radioactive substrate assays²⁷ or on the classical malachite green assay, which measures pyrophosphate and is not specific for dUTPase.²³ No luminescence or fluorescence-based assay specific for this enzyme is yet known. Having access to a simple luminescence assay that functions *in vitro*, and also in cell and tissue lysates, would greatly simplify biological studies of the enzyme's cellular roles. Moreover, such an assay could be clinically useful, by assisting in searches for new inhibitors of these pathways and by aiding in patient prognosis for cancers treated with fluoropyrimidines, as well as in patient selection for targeted therapeutics.

Here we describe the development of a luminescence probe that specifically reports on dUTPase activity. The DeoxyUridine ATP-Linked (DUAL) probe is a tetraphosphate-bridged chimera of dUTP and ATP. We report that the enzyme accepts the probe as an efficient substrate and, upon interphosphate bond hydrolysis, releases ATP, which is detected sensitively by luciferase luminescence. This allows the detection of dUTPase activity in real time *in vitro*. Moreover, data reveal that the DUAL probe can be used to measure and compare activity in cell lysates, and we use it to report for the first time the levels of dUTPase activity in several different cell lines.

RESULTS

Design and Synthesis of the DUAL Probe.

The design of the DUAL probe (Figure 1) incorporates dUTP and ATP in a single tetraphosphate-bridged 5',5'-dinucleotide. Substrate recognition for the dUTPase enzyme is provided by the deoxyuridine portion of the compound, which for signaling purposes is linked to adenosine via the tetraphosphate linkage. In principle, the dUTPase enzyme could recognize the deaminated nucleotide and its first three phosphates as a normal substrate, with the remaining terminal adenosine monophosphate potentially left outside the binding pocket.¹¹ Enzymatic cleavage activity would cleave the phosphoanhydride linkage between α and β phosphates nearest to deoxyuridine, directly analogous to the native substrate mechanism.¹² However, instead of releasing pyrophosphate, ATP would instead be produced. Luciferase enzyme could then be used to generate luminescence signals in the presence of luciferin, providing a light signal directly proportional to moles of substrate

consumed. This dinucleotide adopts design principles incorporated in recently reported ATP-linked chimeras of other deoxynucleotides: ATP-linked variants of the canonical deoxynucleotides have been used to report on reverse transcriptase activity in detection of RNA,²⁸ and ATP conjugates of 8-oxo-dGTP and of deoxyinosine have been described recently for detection of the activity of the MTH1 and ITPA enzymes.^{29,30}

While the theoretical design of this chimeric substrate is relatively straightforward, a number of questions remained about its performance. These included (a) whether the human dUTPase enzyme would in fact accept this chimera as a substrate despite the large AMP residue potentially interfering at its terminus; (b) whether luciferase would accept the AMP end of the chimera as a substrate, thereby short-circuiting the design and obviating its utility; (c) whether enzymatic buffer conditions could be found that are compatible both with dUTPase and luciferase enzymatic activities; and (d) whether the probe was sufficiently selective and sensitive to measure variable dUTPase activity in cell lysates with constitutive enzyme levels.

Synthesis of the DUAL chimera was carried out by EDC-mediated coupling of ATP and deoxyuridine monophosphate.³¹ After cation exchange, coupling yielded the DUAL dinucleotide, which was purified by high performance liquid chromatography. The DUAL probe was characterized by mass spectrometry and NMR spectra (see Methods and Supporting Information (SI) for data).

Coupling of dUTPase and Luciferase Reporting.

First, we tested whether firefly luciferase could recognize the ATP moiety directly in the DUAL probe, which, if it were the case, would obviate its utility by generating signals whether or not dUTPase activity was present. Experiments performed in luciferase buffer showed activity that was less than 0.1% that of native ATP (actual value $0.99 \pm 0.02\%$). It is not clear whether the small signal observed arises from weak enzymatic activity with this unnatural substrate, or whether the signal is simply a residual enzymatic signal from a very small amount of ATP contaminant from the probe purification. While no ATP was detectable in the preparation by NMR and HPLC, we cannot rule out very small contaminating levels of the natural nucleotide. In any case, the experiments confirm that luciferase is highly selective for ATP over this terminal conjugate of ATP and that DUAL is a very poor substrate. This is consistent with firefly luciferase active site structure,³² which conforms around the ATP, placing the terminal γ -phosphate near to the luciferin acceptor. Appending a large nucleotide would not be expected to be accommodated efficiently in this pocket.

To evaluate the ability of dUTPase enzyme to recognize the dUTP portion of the probe and discriminate between it and other related nucleotides, we next incubated the enzyme with five different ATP-linked nucleotides (Figure 2B). To measure signals, we performed the reactions in 1:1 mixed dUTPase reaction buffer and kinase-Glo reaction buffer, which proved to be compatible both with dUTPase and luciferase activity. The buffer contained both dUTPase (2 nM) and firefly luciferase, as well as luciferin substrate and ATP-linked nucleotide (20 μ M). Reactions (typically 20 μ L total volume) were then initiated by addition of dUTPase enzyme, and the reactions were monitored at 30 °C in a luminescence plate reader. The results showed signals increasing clearly over time as the luciferase signal is

generated in the presence of the DUAL substrate. In contrast, little or no signal was seen for similar conjugates of dA, dC, dT, and dG (Figure 2B). Thus, the enzyme shows a clear and strong preference for deoxyuridine in a chimera, as described previously for native triphosphate substrates,³³ and is highly selective for the DUAL probe among these five structurally varied nucleotide substrates. We note that one of them (DTAL) differs from the DUAL probe by only a single methyl group, underlining the high specificity of this enzyme for uracil-containing substrates.

Enzyme Substrate Capabilities.

Next, we evaluated the efficiency of DUAL as a substrate for dUTPase enzyme. Measurements of enzyme rate as a function of DUAL concentration allowed us to quantify Michaelis–Menten parameters for the substrate (Figure 3). The results show that DUAL is a relatively efficient substrate, with K_m very close to that reported for the native substrate.³⁴ This establishes near-native binding affinity for the modified substrate; examination of the published structure¹¹ suggests that the terminal adenosine monophosphate could be accommodated outside the substrate binding pocket in the trimeric enzyme complex without severe interactions. The chemical cleavage of the DUAL substrate is lower than that of the native substrate by a relatively small factor of 7-fold. Thus, overall we conclude that DUAL is an efficient substrate for this enzyme.

Selectivity and Sensitivity Measurements.

We proceeded to evaluate the performance of the DUAL probe further, by testing its selectivity and sensitivity. Data are presented in Figure 4A. Testing the DUAL probe with related nucleotide pool sanitation enzymes MTH1³⁵ and ITPA³⁶ showed little or no signal under conditions that yield a robust luminescence signal from dUTPase enzyme, confirming enzyme selectivity of the probe among these related enzymes. We confirmed that these other two enzymes were indeed active with separate assays of their activity (data not shown).

To test sensitivity of the DUAL probe, we quantified the luciferase signal under identical conditions but with increasingly small quantities of dUTPase enzyme (Figure 4B). We found that dUTPase activity could be detected down to 25 pg of enzyme or less. This high degree of sensitivity likely reflects the fact that the K_m of the enzyme is close to that of the native substrate, and thus dUTPase retains good binding affinity with the DUAL probe. Moreover, even at concentrations well below saturation, the signal generation remains detectable because the k_{cat} of DUAL is not far below that of native dUTP. Overall, the sensitivity is sufficient to measure differences in dUTPase activity in cultured cells (see below).

Cell Lysate dUTPase Measurements.

We proceeded to test the ability of the DUAL probe/luciferase assay to quantify differences in dUTPase activity in cell lysates. Because cells contain high concentrations of ATP that would interfere with signals, we first depleted ATP from the lysates using a gel filtration spin columns and washing²⁹ (see the Experimental Methods section). The macromolecular cellular fraction was then resuspended and incubated with DUAL and luciferase for measurement of dUTPase activity by luminescence. Our first experiments were carried out in HEK293T cells with siRNA suppression of dUTPase.³⁷ After siRNA treatment (24-h

post-transfection), we measured DUAL signals as compared with untreated lysate (Figure 5). The data show a lower signal in the siRNA-treated cells (40% of control), consistent with suppression of dUTPase expression. Separate measurement of dUTPase protein levels by Western blots (Figure 5B) revealed virtually the same level of suppression, thus suggesting that DUAL accurately reflects activity of dUTPase enzyme without high levels of background signal.

Next, we proceeded to employ the DUAL assay to measure dUTPase signals in eight other cell lines, including cell lines from breast, lung, bone, colorectal, cervical, and brain tumor origins (Figure 6A). We found a range of relative activities that varied by a factor of 7 from the highest (HEK293T) to the lowest (HT29 and U87MG). For comparison, we also measured dUTPase protein quantities by Western blots in the same cell lines (Figure 6B). Overall, the qualitative agreement was excellent between the two methods (Figure 6C), and the Western blot-measured protein quantities were again highest in HEK293T and lowest in HT29 and U87MG cells. However, closer examination of the DUAL signals compared with protein quantities reveals some significant differences. HEK293T activity signals are higher than expected from protein quantity, while activity signals from H1299 and A549 cells are lower than expected. We suggest that these deviations may reflect possible upregulation and downregulation of activity, such as occurs by varied levels of phosphorylation in the nuclear dUTPase fraction.^{13–15} Future work will be needed to test this possibility.

DISCUSSION

We have described a new molecular probe, DUAL, that specifically reports on human dUTPase activity. The probe is prepared conveniently in a single synthetic step and can be isolated in over 30% yield after purification. The method employing the probe is simple and can be carried out in one tube in a standard luminescence plate reader. It yields real-time measurements of activity and is sensitive enough to measure activity in ATP-depleted cell lysates. We further employ it here to quantify relative dUTPase activity levels in several common cell lines.

The results further establish the generality and utility of ATP-linked nucleotides in luminescence-based quantification of enzyme activities. Our initial studies using this strategy involved canonical deoxynucleotides linked to a terminal ATP residue.²⁸ These have been useful for direct reporting on DNA polymerase activity in one- and two-tube assays and have shown high sensitivity in detection of siRNAs and long RNAs as well. More recently, we described an ATP-linked chimera of 8-oxodeoxyguanosine;²⁹ this “ARGO” probe has shown utility in quantifying cellular activity of the nucleotide surveillance enzyme MTH1, also a clinical target in cancer, and is now in use in multiple laboratories. Since several enzymes are known to act on modified and damaged nucleotides in the cellular nucleotide pool,³⁸ one can envision further extensions and applications of this molecular strategy in the future.

Previous *in vitro* measurements of dUTPase activity have commonly employed the malachite green (MG) assay, which measures pyrophosphate colorimetrically.²³ The sensitivity of the MG assay for dUTPase specifically has not been reported to our

knowledge, but its sensitivity for pyrophosphate is reported to be in the nanomole range (micromolar concentrations).³⁹ By comparison, the sensitivity of the DUAL probe assay is high (25 pg of protein can be detected) since the background signal from luciferase chemiluminescence is very low. In addition, the MG assay is not specific for dUTPase, as it detects a product that is produced by many enzymes, including other nucleotide surveillance enzymes.³⁷ In contrast, the DUAL substrate contains deoxyuridine structure, conferring high-affinity enzyme recognition and structural selectivity.

Our early experiments employing DUAL for evaluating cellular dUTPase activity are promising and suggest general utility for this purpose. Direct measurement of activity may be more informative and relevant in many applications than indirect measurements such as mRNA levels or protein quantity by Western blot. Moreover, the current assay is faster and simpler than those two methods. To our knowledge, there exist no prior reports in the literature of dUTPase activity measurements from cell lines or tissues, and we suggest that the current method can greatly enhance our knowledge of the biology of the enzyme, its regulation, and its clinical response and prognostic power.

The NUDIX hydrolase family of enzymes, to which dUTPase is related, exhibits significant overlapping activity for some substrates, and non-dUTPase members of the family could potentially contribute to some of the signal seen here.^{37,40} The FHIT enzyme is a diribonucleotide hydrolase with activity in cleaving Ap4A;⁴¹ it is not yet known whether it can accept deoxynucleotides or nucleobases other than A in its dinucleotide substrate. The NUDT2 enzyme, which also displays dinucleotide hydrolase activity, is better studied for substrate preferences, and early data suggest that it can cleave Ap4, Ap4A, and to a lesser extent, Ap4dT.⁴² This last substrate is close in structure to our DUAL substrate, which suggests the possibility of some overlapping activity and background signal in cell lysates. Further studies with these and other NUDIX-family enzymes will be instructive on these points.

The DUAL probe measurements of dUTPase activity in cell lines may be useful for future studies of this enzyme, enabling researchers to choose high- and low-activity lines for study and comparison. The DUAL probe is likely to be generally useful in measuring this activity in other cells and tissues as well. It will be of special interest in the future to employ this probe in assessment of deoxyuridine triphosphatase activity in primary patient cells and tissues, as it may well be of clinical relevance to assess patients for cancer prognosis¹⁷ or for dUTPase inhibition therapy²⁴ based on the enzyme's activity in biopsy specimens. These initial results suggest that the DUAL assay is a promising candidate for that application.

EXPERIMENTAL METHODS

General.

¹H and ³¹P NMR spectra were recorded on a Varian Inova 500 MHz NMR spectrometer. Semipreparative 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. The bioluminescence signal was recorded by a microplate fluorometer (Fluoroskan Ascent,

Thermal). Chemicals were purchased from commercial suppliers (Sigma Aldrich or Alfa Aesar) unless otherwise indicated. Recombinant human dUTPase protein was purchased from Abcam (Cambridge, USA). The Kinase-Glo Luminescent Kinase Assay kit was used for the bioluminescence assay. A Bio-Rad Protein Assay Kit was used for determination of the protein concentration of cell lysates.

Synthesis of the DUAL Probe.

The sodium salts of dUTP and AMP were dissolved in distilled deionized water and converted to their free acids 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 white powder. The lyophilized powders of the nucleotides were coevaporated with anhydrous DMF twice and stored under high vacuum for 3 h before the subsequent coupling reaction. For coupling, the tetrabutylammonium salt of dUTP (40 mg, 30 μ mol) was dissolved in 1 mL of anhydrous DMF. To this solution was added carbonyldiimidazole (CDI, 25 mg, 150 μ mol), and the mixture was stirred at room temperature for 5 h, after which 20 μ L of MeOH was added to quench the reaction. All solvents were removed under high vacuum, and the residue was redissolved in 1 mL of anhydrous DMF. AMP tetrabutylammonium salt (50 mg, 50 μ mol) was added as a solution in 1 mL of DMF with anhydrous $MgCl_2$ (5 mg). The mixture was stirred for 72 h at room temperature. After this, the product was precipitated by the addition of acetone (10 mL). The chimeric nucleotide 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, eluted 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 35%. 1H NMR (D_2O , 500 MHz, NH_4^+ form): δ 8.38 (s, 1H), 8.09 (s, 1H), 7.66 (d, J = 8.1 Hz, 1H), 6.08 (t, J = 6.8 Hz, 1H), 5.97 (d, J = 6.1 Hz, 1H), 5.68 (d, J = 8.0 Hz, 1H), 4.63 (t, J = 5.7 Hz, 1H), 4.43 (ddd, J = 9.0, 5.8, 3.6 Hz, 2H), 4.25 (t, J = 3.0 Hz, 1H), 4.20–4.07 (m, 2H), 4.06–3.95 (m, 3H), 2.37–1.91 (m, 2H). ^{31}P NMR (D_2O , 500 MHz, NH_4^+ form): δ –10.24 – –10.75 (m), –21.56 (m). HRMS: calculated for $C_{19}H_{26}N_7O_{20}P_4$ (M – H) – 796.0191, found 796.0166. See the SI for spectra.

Enzyme and Luciferase Methods.

For a two-tube reaction, the standard 20 μ L reaction buffer contained 70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, pH 7.6, 40 μ M DUAL nucleotide, and varied amounts of dUTPase as described. After 1 h incubation at 30 °C, 5 μ L of this reaction solution was added to 20 μ L of Kinase-Glo Luminescent Kinase Assay reaction solution (prepared as instructed for the ATP determination kit) in a 96 well plate. The bioluminescence signal was recorded at 1 min intervals over 1 h by a microplate fluorometer.

For a one-tube reaction, 10 μ L of reaction buffer (70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) containing 40 μ M DUAL nucleotide was mixed directly with 10 μ L of Kinase-Glo Luminescent Kinase Assay reaction solution, and then 2 nM dUTPase (1 μ L) was added from a 40 nM stock. The reaction was monitored at 1 min intervals over 2 h by a microplate luminometer.

Enzyme Kinetics.

A Michaelis–Menten curve was generated by adding dUTPase (2 nM) in 40 μL of 70 mM Tris-HCl (pH 7.6), 10 mM MgCl_2 , 5 mM DTT, and various amounts of DUAL (0.6–40 μM) at 30 °C for 3 min. Then 5 μL of the reaction solution was added to 20 μL of Kinase-Glo Luminescent Kinase Assay reaction solution to calculate the amount of ATP generated. Reactions were performed with three replicates. The velocity was plotted as a function of DUAL concentration and fit with the Michaelis–Menten equation to obtain the kinetic parameters, V_{max} and K_{m} . The k_{cat} values were calculated by dividing V_{max} by the concentration of dUTPase used.

Cell Lysate dUTPase Measurements.

H1299 cells (ATCC CRL-5803) were grown in ATCC-formulated RPMI-1640 Medium; A549 cells (ATCC) were grown in F-12K Medium (Kaighn's Modification of Ham's F-12 Medium, ATCC); HT-29 (ATCC) and U2OS cells (ATCC) were grown in McCoy's 5A Medium; and HeLa (ATCC), MCF7 (ATCC), and U87 MG (ATCC) cells were grown in ATCC-formulated Eagle's Minimum Essential Medium. HEK293T cells were grown in ATCC-formulated Dulbecco's Modified Eagle's Medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin, and cells were grown in a humidified incubator at 37 °C with 5% CO_2 . 6×150 mm plates of cells were grown to 90% confluence. The cells were harvested, and the preparation of cell lysates was the same as the published method.²⁹ The cell lysate was transferred to prechilled tubes and kept at –80 °C until use. Protein concentrations in these cell lysates were measured by a Bio-Rad Protein Assay Kit (Bradford method) following the manufacturer's instructions.

The HEK293T cells were seeded in a 75 cm^2 flask at a 60% confluence level and transfected with approximately 250 pmol of dUTPase siRNA (h) (sc-62242 Santa Cruz Biotechnology) or control siRNAs (sc-44230) using Lipofectamine RNAiMAX Reagent. After 24 h incubation, the cells were harvested, and cell lysate was prepared as described above.

Cell lysates were diluted to 0.5 $\mu\text{g}/\mu\text{L}$; 1 μL of each diluted cell lysate was added to 20 μL of dUTPase reaction buffer containing 70 mM Tris-HCl (pH 7.6), 10 mM MgCl_2 , 5 mM DTT, and 40 μM DUAL nucleotide. After 0.5 h incubation at 30 °C, 5 μL of this reaction solution was added to 20 μL of Kinase-Glo Luminescent Kinase Assay reaction solution to calculate the amount of ATP generated by the dUTPase reaction. All data were the averages from three replicates.

Western Blots.

Thirty μg of the total protein lysate was mixed with 5x Laemmli Buffer (5% SDS, 50% Glycerol, 0.1% Bromophenol Blue, 250 mM Tris-HCl, pH 6.8) and loaded on a 4–20% Bis-Tris Protein Gel (Invitrogen), next to Amersham Full Range Rainbow Recombinant Protein Molecular Weight Markers (GE Healthcare). Electrophoresis was run in 1x NuPage MES Running Buffer (Life Technologies) for 1 h at room temperature. Protein transfer was done with a BlotMini Blot Module (Life Technologies), using 0.45 μm GE Healthcare Amersham Protran Supported NC Nitrocellulose Membranes (GE Healthcare), in 1x NuPage Transfer

Buffer (Life Technologies) with the addition of 10% ethanol (Sigma-Aldrich), 10 V, for 1 h, at room temperature. Membranes were blocked using 5% skimmed milk in PBST buffer (0.1% Tween20 in 1x PBS pH7.4, Gibco) for 30 min at room temperature with constant rocking. Then, the membrane was incubated with primary antibodies anti-dUTPase (1:500, Proteintech 13740-1-AP) and anti- β Actin (1:2000, β -Actin Antibody, Santa Cruz Biotechnology, sc-47778) in 5% skimmed milk in PBST buffer for 16 h at 4 °C with constant rocking. The next day, membranes were washed three times in fresh PBST buffer and incubated for 1 h with a mixture of the secondary antibodies (1:10 000, LI-COR; IRDye 800CW, 925–32213; IRDye, 925–68072) in PBST buffer at room temperature in the dark with constant rocking. The membranes were further washed twice for 5 min using PBST buffer, and then one additional wash (5 min) was done in PBS, at room temperature in the dark with rocking. Membranes were scanned and signal measured using the Odyssey CLx Imaging System. The analysis was performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature* 362, 709–715. [PubMed: 8469282]
- (2). Brynolf K, Eliasson R, and Reichard P (1978) Formation of Okazaki fragments in polyoma DNA synthesis caused by misincorporation of uracil. *Cell* 13, 573–580. [PubMed: 207436]
- (3). Grogan BC, Parker JB, Guminski AF, and Stivers JT (2011) Effect of the thymidylate synthase inhibitors on dUTP and TTP pool levels and the activities of DNA repair glycosylases on uracil and 5-fluorouracil in DNA. *Biochemistry* 50, 618–627. [PubMed: 21222484]
- (4). Andersen S, Heine T, Sneve R, König I, Krokan HE, Epe B, and Nilsen H (2005) Incorporation of dUMP into DNA is a major source of spontaneous DNA damage, while excision of uracil is not required for cytotoxicity of fluoropyrimidines in mouse embryonic fibroblasts. *Carcinogenesis* 26, 547–555. [PubMed: 15564287]
- (5). Friedberg E, Walker G, Siede W, Wood R, Schultz R, and Ellenberger T (1995) *DNA Repair and Mutagenesis* American Society for Microbiology Press, Washington, DC.
- (6). Canman CE, Lawrence TS, Shewach DS, Tang HY, and Maybaum J (1993) Resistance to fluorodeoxyuridine-induced DNA damage and cytotoxicity correlates with an elevation of deoxyuridine triphosphatase activity and failure to accumulate deoxyuridine triphosphate. *Cancer Res* 53, 5219–5224. [PubMed: 8221659]
- (7). Wilson PM, Fazzzone W, LaBonte MJ, Deng J, Neamati N, and Ladner RD (2008) Novel opportunities for thymidylate metabolism as a therapeutic target. *Mol. Cancer Ther* 7, 3029–3037. [PubMed: 18790783]
- (8). Abolhassani N, Iyama T, Tsuchimoto D, Sakumi K, Ohno M, Behmanesh M, and Nakabeppu Y (2010) NUDT16 and ITPA play a dual protective role in maintaining chromosome stability and cell growth by eliminating dIDP/IDP and dITP/ITP from nucleotide pools in mammals. *Nucleic Acids Res* 38, 2891–2903. [PubMed: 20081199]

- (9). Vértessy BG, and Tóth J (2009) Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases. *Acc. Chem. Res* 42, 97–106. [PubMed: 18837522]
- (10). McIntosh EM, Ager DD, Gadsden MH, and Haynes RH (1992) Human dUTP pyrophosphatase: cDNA sequence and potential biological importance of the enzyme. *Proc. Natl. Acad. Sci. U. S. A* 89, 8020–8024. [PubMed: 1325640]
- (11). Mol CD, Harris JM, McIntosh EM, and Tainer JA (1996) Human dUTP pyrophosphatase: uracil recognition by a β hairpin and active sites formed by three separate subunits. *Structure* 4, 1077–1092. [PubMed: 8805593]
- (12). Tóth J, Varga B, Kovács M, Málnási-Csizmadia A, and Vértessy BG (2007) Kinetic mechanism of human dUTPase, an essential nucleotide pyrophosphatase enzyme. *J. Biol. Chem* 282, 33572–33582. [PubMed: 17848562]
- (13). Ladner RD, and Caradonna SJ (1997) The human dUTPase gene encodes both nuclear and mitochondrial isoforms differential expression of the isoforms and characterization of a cDNA encoding the mitochondrial species. *J. Biol. Chem* 272, 19072–19080. [PubMed: 9228092]
- (14). Caradonna S, and Muller-Weeks S (2001) The Nature of Enzymes Involved in Uracil-DNA Repair: Isoform Characteristics of Proteins Responsible for Nuclear and Mitochondrial Genomic Integrity I. *Curr. Protein Pept. Sci* 2, 335–347. [PubMed: 12369930]
- (15). Strahler JR, Zhu X-X, Hora N, Wang YK, Andrews PC, Roseman NA, Neel JV, Turka L, and Hanash SM (1993) Maturation stage and proliferation-dependent expression of dUTPase in human T cells. *Proc. Natl. Acad. Sci. U. S. A* 90, 4991–4995. [PubMed: 8389461]
- (16). Pearl LH, Schierz AC, Ward SE, Al-Lazikani B, and Pearl FM (2015) Therapeutic opportunities within the DNA damage response. *Nat. Rev. Cancer* 15, 166–180. [PubMed: 25709118]
- (17). Takatori H, Yamashita T, Honda M, Nishino R, Arai K, Yamashita T, Takamura H, Ohta T, Zen Y, and Kaneko S (2010) dUTP pyrophosphatase expression correlates with a poor prognosis in hepatocellular carcinoma. *Liver Int* 30, 438–446. [PubMed: 19968781]
- (18). Ladner RD, Lynch FJ, Groshen S, Xiong YP, Sherrod A, Caradonna SJ, Stoehlmacher J, and Lenz HJ (2000) dUTP nucleotidohydrolase isoform expression in normal and neoplastic tissues: association with survival and response to 5-fluorouracil in colorectal cancer. *Cancer Res* 60, 3493–3503. [PubMed: 10910061]
- (19). Chano T, Mori K, Scotlandi K, Benini S, Lapucci C, Manara MC, Serra M, Picci P, Okabe H, and Baldini N (2004) Differentially expressed genes in multidrug resistant variants of U-2 OS human osteosarcoma cells. *Oncol. Rep* 11, 1257–1263. [PubMed: 15138564]
- (20). Wilson PM, LaBonte MJ, Lenz H-JD, Mack PC, and Ladner RD (2012) Inhibition of dUTPase induces synthetic lethality with thymidylate synthase-targeted therapies in non-small cell lung cancer. *Mol. Cancer Ther* 11, 616–280. [PubMed: 22172489]
- (21). Miyahara S, Miyakoshi H, Yokogawa T, Chong KT, Taguchi J, Muto T, Endoh K, Yano W, Wakasa T, and Ueno H (2012) Discovery of a novel class of potent human deoxyuridine triphosphatase inhibitors remarkably enhancing the antitumor activity of thymidylate synthase inhibitors. *J. Med. Chem* 55, 2970–2980. [PubMed: 22339362]
- (22). Miyakoshi H, Miyahara S, Yokogawa T, Endoh K, Muto T, Yano W, Wakasa T, Ueno H, Chong KT, and Taguchi J (2012) 1, 2, 3-Triazole-containing uracil derivatives with excellent pharmacokinetics as a novel class of potent human deoxyuridine triphosphatase inhibitors. *J. Med. Chem* 55, 6427–6437. [PubMed: 22715973]
- (23). Hagenkört A, Paulin CB, Desroses M, Sarno A, Wiita E, Mortusewicz O, Koolmeister T, Loseva O, Jemth AS, and Almlöf I (2017) dUTPase inhibition augments replication defects of 5-Fluorouracil. *Oncotarget* 8, 23713–23726. [PubMed: 28423595]
- (24). Saito K, Nagashima H, Noguchi K, Yoshisue K, Yokogawa T, Matsushima E, Tahara T, and Takagi S (2014) First-in-human, phase I dose-escalation study of single and multiple doses of a first-in-class enhancer of fluoropyrimidines, a dUTPase inhibitor (TAS-114) in healthy male volunteers. *Cancer Chemother. Pharmacol* 73, 577–583. [PubMed: 24452393]
- (25). Hizi A, and Herzig E (2015) dUTPase: the frequently overlooked enzyme encoded by many retroviruses. *Retrovirology* 12, 70. [PubMed: 26259899]
- (26). Baragaña B, McCarthy O, Sanchez P, Bosch-Navarrete C, Kaiser M, Brun R, Whittingham JL, Roberts SM, Zhou XX, and Wilson KS (2011) β -Branched acyclic nucleoside analogues as

inhibitors of *Plasmodium falciparum* dUTPase. *Bioorg. Med. Chem* 19, 2378–2391. [PubMed: 21411327]

- (27). Prangishvili D, Klenk HP, Jakobs G, Schmiechen A, Hanselmann C, Holz I, and Zillig W (1998) Biochemical and phylogenetic characterization of the dUTPase from the archaeal virus SIRV. *J. Biol. Chem* 273, 6024–6029. [PubMed: 9497317]
- (28). Ji D, Mohsen MG, Harcourt EM, and Kool ET (2016) ATP-Releasing Nucleotides: Linking DNA Synthesis to Luciferase Signaling. *Angew. Chem., Int. Ed* 55, 2087–2091.
- (29). Ji D, Beharry AA, Ford JM, and Kool ET (2016) A Chimeric ATP-Linked Nucleotide Enables Luminescence Signaling of Damage Surveillance by MTH1, a Cancer Target. *J. Am. Chem. Soc* 138, 9005–9008. [PubMed: 27413803]
- (30). Ji D, Stepchenkova EI, Cui J, Menezes MR, Pavlov YI, and Kool ET (2017) Measuring Deaminated Nucleotide Surveillance Enzyme ITPA Activity with an ATP-releasing Nucleotide Chimera. *Nucleic Acids Res* 45, 11515–11524. [PubMed: 29036687]
- (31). Sims PA, Greenleaf WJ, Duan H, and Xie XS (2011) Fluorogenic dnA sequencing in Pdms microreactors. *Nat. Methods* 8, 575–583. [PubMed: 21666670]
- (32). Conti E, Franks NP, and Brick P (1996) Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure* 4, 287–298. [PubMed: 8805533]
- (33). Ladner RD, McNulty DE, Carr SA, Roberts GD, and Caradonna SJ (1996) Characterization of distinct nuclear and mitochondrial forms of human deoxyuridine triphosphate nucleotidohydrolase. *J. Biol. Chem* 271, 7745–7751. [PubMed: 8631816]
- (34). Climie S, Lutz T, Radul J, Sumnersmith M, Vandenberg E, and McIntosh E (1994) Expression of trimeric human dUTP pyrophosphatase in *Escherichia coli* and purification of the enzyme. *Protein Expression Purif* 5, 252–258.
- (35). Nakabeppu Y, Ohta E, and Abolhassani N (2017) MTH1 as a nucleotide pool sanitizing enzyme: Friend or foe? *Free Radical Biol. Med* 107, 151–158. [PubMed: 27833032]
- (36). Simone PD, Pavlov YI, and Borgstahl GE (2013) ITPA (inosine triphosphate pyrophosphatase): from surveillance of nucleotide pools to human disease and pharmacogenetics. *Mutat. Res., Rev. Mutat. Res* 753, 131–146.
- (37). Studebaker AW, Lafuse W, Kloesel R, and Williams M (2005) Modulation of human dUTPase using small interfering RNA. *Biochem. Biophys. Res. Commun* 327, 306–310. [PubMed: 15629463]
- (38). Tsuchimoto D, Iyama T, Nonaka M, Abolhassani N, Ohta E, Sakumi K, and Nakabeppu Y (2010) A comprehensive screening system for damaged nucleotide-binding proteins. *Mutat. Res., Genet. Toxicol. Environ. Mutagen* 703, 37–42.
- (39). Lanzetta PA, Alvarez LJ, Reinach PS, and Candia OA (1979) An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem* 100, 95–97. [PubMed: 161695]
- (40). Carmi-Levy I, Yannay-Cohen N, Kay G, Razin E, and Nechushtan H (2008) Diadenosine tetraphosphate hydrolase is part of the transcriptional regulation network in immunologically activated mast cells. *Mol. Cell. Biol* 28, 5777–5784. [PubMed: 18644867]
- (41). Barnes LD, Garrison PN, Siprashvili Z, Guranowski A, Robinson AK, Ingram SW, Croce CM, Ohta M, and Huebner K (1996) Fhit, a Putative Tumor Suppressor in Humans, Is a Dinucleoside 5', 5'''-P1, P3-Triphosphate Hydrolase. *Biochemistry* 35, 11529–11535. [PubMed: 8794732]
- (42). Carreras-Puigvert J, Zitnik M, Jemth A-S, Carter M, Unterlass JE, Hallström B, Loseva O, Karem Z, Calderón-Montaña JM, Lindskog C, Edqvist PH, Matuszewski DJ, Ait Blal H, Berntsson RPA, Haggblad M, Martens U, Studham M, Lundgren B, Wahlby C, Sonnhämmer ELL, Lundberg E, Stenmark P, Zupan B, and Helleday T (2017) A comprehensive structural, biochemical and biological profiling of the human NUDIX hydrolase family. *Nat. Commun* 8, 1541. [PubMed: 29142246]

Figure 1. Structure of the DUAL probe and mechanism for signaling dUTPase activity. Cleavage between α and β phosphate groups nearest deoxyuridine (deaminated base shown in blue) releases ATP, which generates a luminescence signal in the presence of luciferase and luciferin.

Figure 2.

Substrate capabilities of DUAL and other ATP-linked nucleotides. A. DUAL is a very poor direct substrate for firefly luciferase, as shown by relative signals of DUAL nucleotide and ATP in equimolar amounts. B. dUTPase enzyme shows high selectivity for the DUAL probe over ATP-linked versions of the four canonical deoxynucleotides (dG, dC, dA, dT).²⁸

Figure 3.

Enzyme substrate parameters of DUAL. At left is a saturation plot, showing the reaction rate as a function of DUAL concentration; at right are Michaelis–Menten parameters as compared with values reported for the native dUTP substrate.³³

Figure 4.

Performance of the DUAL probe. A. Selectivity of DUAL for the dUTPase enzyme relative to two other nucleotide sanitation enzymes (MTH1, ITPA). B. Sensitivity of DUAL as measured by linearity of luminescence response at increasingly lower enzyme concentrations.

Figure 5. Measuring siRNA-mediated suppression of dUTPase activity in HEK293T cell lysate. A. Raw luminescence intensity measurements of untreated cells vs siRNA-treated cells (24 h). B. dUTPase protein quantity by Western blot, normalized to β -actin.

Figure 6.

Relative dUTPase activity in eight cell lines measured by the DUAL assay. A. Relative dUTPase activity as luminescence signals from DUAL activity assay. B. Western blot data showing relative protein quantities. C. Correlation plot showing relatively low/downregulated dUTPase activity (relative to protein quantity) in HEK293T cells and high/upregulated activity in H1299 and A549 cell lines.