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An Enzyme-Coupled Microplate Assay for Activity and Inhibition of hmdUMP Hydrolysis by DNPH1

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Abstract

2′-Deoxynucleoside 5′-monophosphate N-glycosidase 1 (DNPH1) hydrolyzes the epigenetically modified nucleotide 5-hydroxymethyl 2′-deoxyuridine 5′-monophosphate (hmdUMP) derived from DNA metabolism. Published assays of DNPH1 activity are low throughput, use high concentrations of DNPH1, and have not incorporated or characterized reactivity with the natural substrate. We describe the enzymatic synthesis of hmdUMP from commercially available materials and define its steady-state kinetics with DNPH1 using a sensitive, two-pathway enzyme coupled assay. This continuous absorbance-based assay works in 96-well plate format using nearly 500-fold less DNPH1 than previous methods. With a Z prime value of 0.92, the assay is suitable for high-throughput assays, screening of DNPH1 inhibitors, or characterization of other deoxynucleotide monophosphate hydrolases.

Graphical Abstract

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Author contributions

A.G.W. is responsible for conceptualization, data curation, formal analysis, methodology, as well as writing and editing of the original manuscript. R.E. was responsible for data curation, formal analysis, and reviewing and editing of the final manuscript draft. V.L.S. was responsible for conceptualization, funding acquisition, project administration, supervision, as well as reviewing and editing of the final manuscript draft.

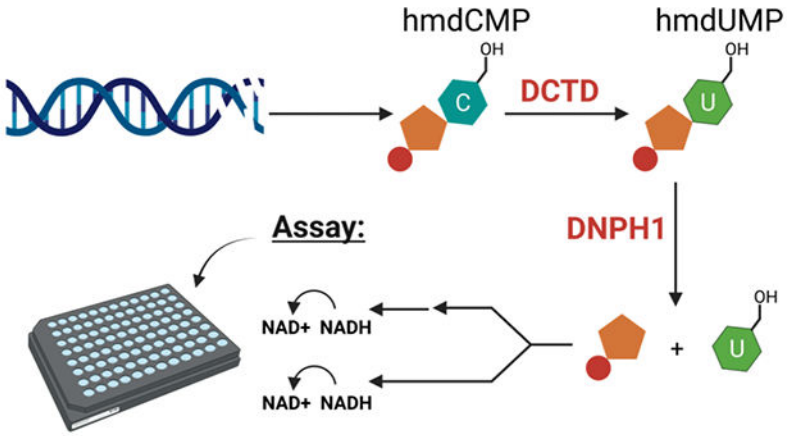
Ethics approval and consent to participate

The manuscript does not contain clinical or trial studies on patients, humans, or animals.

Declaration of competing interest

The authors declare that they have no competing interest.

Appendix A. Supplementary data



Keywords

DNPH1; 5-hydroxymethyl uracil; N-deoxynucleotide glycosidase; pyrimidine salvage; epigenetics; 2-deoxyribose 5-phosphate aldolase

1. Introduction

Covalent modification of nucleotides following DNA replication provides epigenetic signaling for appropriate RNA synthesis and is necessary for the progression of cell division.[1] DNA synthesis requires balanced deoxynucleotide pools with removal of inappropriate deoxynucleotides (like 2′-deoxyuridine 5′-triphosphate, dUTP) or epigenetically modified nucleotides (like 5-hydroxymethyl-dUTP).[2-4] The DNA precursor pool is sustained by an interplay of metabolic pathways that control the production, breakdown, and reutilization of deoxynucleotides.[5, 6] Errors in DNA replication are prevented by a combination of enzymes in *de novo* and salvage pathways to maintain the appropriate equilibrium of deoxynucleotides.[7-9]

The N-glycosidases (EC 3.2.2.x) play a crucial role in regulation of the nucleotide and deoxynucleotide pathways by hydrolyzing N-glycosidic bonds.[10-13] Recently, an enzyme in this family named 2′-deoxynucleoside 5′-monophosphate N-glycosidase 1 (DNPH1, formerly named Rcl), was re-examined and found to have a unique specificity towards deoxyribose nucleotide monophosphate with the epigenetically modified base 5-hydroxymethyl uracil (hmU).[3] The nucleotide 5-hydroxymethyl 2′-deoxyuridine 5′-monophosphate (hmdUMP) is generated from the action of ten-eleven translocation (TET) thymidine dioxygenases.[14] DNA repair activity generates hmdCMP, and cytidine deaminase (DCTD) activity results in an increasing pool of the epigenetically modified pyrimidine deoxynucleotide hmdUMP.[3, 15] DNPH1 has been reported to act on hmdUMP to prevent its incorporation into the precursor pool for DNA synthesis.[3] The inhibition of DNPH1 has the potential to increase the pool of hmdUMP nucleotides in the cell, which can result in incorporation of hmdU into DNA causing mutations.[3] In the context of BRCA-deficient cancers, an increase in hmdU in the DNA will lead to and an increased level of PARP trapping by PARP inhibitor therapeutics.[3] Therefore, DNPH1 is a compelling target for

the sensitization of resistant tumors to PARP inhibition, and the development of DNPH1 inhibitors may allow for the expansion of synthetic lethal therapeutic strategies.

Inhibitor discovery efforts toward DNPH1 have been hampered by the lack of a sensitive assay which is continuous, uses minimal DNPH1, and is capable of high-throughput screening and characterization of the endogenous substrate. DNPH1 was first characterized as a downstream target of c-Myc and general nucleotide hydrolase.[16] The enzyme was shown to react slowly with nearly all deoxynucleotide monophosphates (dNMPs), and was originally reported to display a preference for purines.[16, 17] Reported catalytic efficiencies (k_{cat}/K_M) of dNMPs with human DNPH1 ranged from as low as 1.0 $M^{-1}s^{-1}$ for 5′-thymidylic acid (dTMP) to as high as 35 $M^{-1}s^{-1}$ for 2′-deoxyguanosine 5′-monophosphate (dGMP),[17] and as a result dGMP was the preferred substrate for all kinetic characterizations of DNPH1.[16-18] However, previously published assays of dGMP hydrolysis often used low throughput methods including TLC, HPLC and NMR[3, 16, 17] which separated guanine product from dGMP substrate and required high concentrations of DNPH1 ranging from 1 micromolar for HPLC assay,[3] to 14 micromolar for kinetic characterization by TLC.[18]

Here we describe the chemoenzymatic synthesis of the natural substrate hmdUMP from commercially available starting material, and its characterization by a dual-product enzyme-coupled assay. HmdUMP is described here to have a catalytic efficiency of 52,380 $M^{-1}s^{-1}$, which is a large improvement over what is previously described for dGMP.[17] We also improve a previously reported low-throughput enzyme coupled assay which used 2-deoxy-d-ribose-5-phosphate aldolase (DERA), triose phosphate isomerase (TPI), and α-glycerophosphate dehydrogenase (GPDH) to couple the formation of 2′-deoxyribose 5′-monophosphate (dR5P) from DNPH1 activity to oxidation of NADH (Scheme 1, **path 1**).[16] We increased the sensitivity of the assay by including a second coupled pathway for NADH oxidation using alcohol dehydrogenase to convert the acetaldehyde product of the DERA reaction to ethanol (Scheme 1, **path 2**), doubling the NADH turnover for each turnover of DNPH1. This duplication of 340 nm absorbance loss permits a robust signal-to-noise ratio in the case of substrates that have low micromolar K_M values and require small amounts of product turnover. The imperative use of the natural substrate hmdUMP also allows for optimization of this assay, using only 30 nM DNPH1, making it require 100-fold less enzyme than previous enzyme coupled assay,[16] and 33-467 times less than other low throughput methods of kinetic characterization which required 1-14 micromolar DNPH1 respectively.[3, 17, 18] These improvements permit assays in small reaction volumes allowing for screening in a 96-well plate format. As an inhibition assay, this method is suitable for screening of focused compound libraries, and for detailed kinetic characterization of DNPH1 inhibitors.

2. Materials and Methods

2.1 General Information

pET-28a (+) plasmids containing codon-optimized genes for *Homo sapiens* DNPH1 (21 kDa), *Escherichia coli* DERA (28 kDa), and *Anopheles gambiae* deoxy nucleoside kinase (*Ag*dNK, 28 kDa) were synthesized by GenScript. BL21DE3 *E. coli* for protein expression was purchased from New England Biolabs (NEB). Fisherbrand™ clear 96-well

flat-bottom polystyrene plates were purchased from Fisher Scientific. Coupled enzymes alcohol dehydrogenase (ADH, *S. cerevisiae*, Cat. No. A7011), triose phosphate isomerase (TPI, rabbit muscle, Cat. No. T6258), and α-glycerophosphate dehydrogenase (GPDH, rabbit muscle, Cat. No. G6880) were purchased from Millipore Sigma. 5-Hydroxymethyl 2′-deoxyuridine (hmdU) was purchased from Biosynth (cat. ND08018). 5′-Thymidylic acid (dTMP) and 2′-deoxyguanosine 5′-monophosphate (dGMP) were purchased from Cayman Chemicals. 2′-Deoxycytidine 5′-monophosphate (dCMP) was purchased from Thermofischer. 2′-Deoxyuridine 5′-monophosphate (dUMP) and 2′-deoxyinosine 5′-monophosphate (dIMP) were purchased from Fischer Scientific. 2′-Deoxyadenosine 5′-monophosphate (dAMP), adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP) guanosine 5′-monophosphate (GMP), uridine 5‘-monophosphate (UMP), 2-deoxyribose 5-monophosphate (dR5P), β-NADH, and all materials to prepare buffers were purchased from Millipore Sigma.

2.2 Purification of DNPH1, DERA, and AgdNK

DNPH1 (UniProt: O43598), DERA (UniProt: P0A6L0), and *Ag*dNK (UniProt: Q86LB8) were all expressed in *E. coli* and purified using identical procedures. Purification gels of His_6-DNPH1, His_6-DERA, and His_6-*Ag*dNK are shown in SI Figures 1, 2, and 3 respectively.

pET-28a (+) plasmids containing genes for either DNPH1, DERA, or *Ag*dNK with an N-terminal His_6 tag were transformed into BL21DE3 *E. coli* (NEB), from which single colonies were grown overnight at 37 °C in 50 mL of Superbroth (Fisher Scientific) containing 50 μg/mL of kanamycin. The overnight cultures were used to inoculate 2 L of Superbroth containing 50 μg/mL of kanamycin, which were grown at 37 °C to an OD_{600} of 0.5 and induced by the addition of 1 mM IPTG. Temperature was reduced to 16 °C for 20 hours, after which cells were harvested by centrifugation.

Pelleted cells were resuspended in lysis buffer containing 20 mM Tris pH 7.4 containing 500 mM sodium chloride, 10 mg/mL lysozyme, 0.1% Triton X-100, and 5 mM imidazole, followed by sonication for 10 mins with 10 second pulses. Insoluble materials were discarded after centrifugation for 1 hour at 18,000 rpm and soluble protein was loaded onto a 5 mL HisTrap HP Ni-NTA column (Cytiva). Proteins were eluted from the column using 4 column volumes of 20 mM Tris pH 7.4 containing 500 mM sodium chloride and stepwise increments of 5 to 500 mM imidazole. The desired enzyme fractions were concentrated using a 10 kDa MW cutoff Amicon Ultra concentrator (Millipore) and stored with 10% glycerol at −80 °C. Unit activity of the DERA enzyme was defined by titration of DERA from 1 μM to 63 nM in 2-fold dilutions with 1 U/mL of ADH and 100 μM NADH in 50 mM HEPES pH 7.0. Reactions were initiated with 100 μM dR5P, and turnover of NADH was monitored in a 100 μL well volume at 37 °C using a SpectraMax M5 microplate reader detecting 340 nm absorbance. Enzyme rate was expressed as μmol product formed/min, plotted against μg DERA added, and fit to a linear trendline (SI Figure 4). 2.77 μg (1 μM in 100 μL) of DERA is equivalent to 1 mU, where one unit is defined by the turnover of 1 μmol of substrate per minute.

Plasmids used for expression of DNPH1, DERA and *Agd*NK will be deposited in Addgene to permit ready access.

2.3 Enzymatic Synthesis of hmdUMP

hmdUMP was synthesized by 5′-phosphorylation of hmdU (Biosynth) by *Ag*dNK. 1 mL reaction mixtures contained 50 mM Tris buffer pH 7.5, 10 mM magnesium chloride, 50 mM potassium chloride, 1 mM hmdU, 0.2 mM ATP, 2 mM phosphoenolpyruvate (PEP), 2 U pyruvate kinase (rabbit muscle, Roche Cat. No. 10128155001) and 1 μM *Ag*dNK. After overnight incubation at 24 °C, hmdUMP was purified using HPLC (Waters Empower 2 software (version 2154)) with a 5 mL HiTrap Q FF strong anion-exchange column (Cytiva). Eluent was a gradient of Milli Q water (solvent A) and 0.5 M ammonium formate (solvent B). The injection volume was 500 μL. The flow rate was 1 mL/min, and the gradient was 100% A for 5 min; 100% A to 100% B over 20 min; 100% B for 5 min; 100% B to 100% A over 5 min; 100% A for 5 min. Elution was monitored by a UV detector at 261 nm. Purified hmdUMP was then lyophilized. The solid was dissolved in 3 mL of water, frozen, and lyophilized to remove residual ammonium formate. Solid hmdUMP (ammonium salt) was stored at −80 °C. The purity was confirmed by NMR and mass spectrometry. ^{1}H NMR (600 MHz, D_2O) δ 2.26 (m, 2H), 3.79 (m, 1H) 3.86 (m, 1H), 4.02 (m, 1H), 4.23 (s, 2H), 4.42 (m, 1H), 6.16 (dd, 1H, J = 1.7 Hz), 7.92 (s, 1H). ^{13}C NMR (150 MHz, D_2O) δ 38.72, 56.52, 61.06, 70.89, 85.51, 86.16 (d, J = 7.5 Hz), 113.91, 139.87, 151.10, 165.07. HRMS calculated for $C_{10}H_{15}KN_2O_9P^+$ $(M+K)^+$ 377.0147, Found, 377.0144. The concentration of the solution was quantitated by the molar absorption e_{261} nm = 10.1 mM^{-1} cm^{-1}.[19]

2.4 DNPH1 Kinetic Assays

2.4.1 Titration of Coupled Enzymes—Purified DNPH1 was diluted to 30 nM in an assay mixture containing 50 mM HEPES pH 7.0 with 200 μM NADH and without one of either 10 mU/mL (1 μM) purified DERA, 1 U/mL ADH, 1 U/mL TPI, or 1 U/mL GPDH. In a 96-well plate, the missing enzyme was titrated to a final concentration ranging from 1 U/mL (or 10 mU/mL for purified DERA) to a lower limit of 51 μU/mL (or 0.51 μU/mL DERA) in 3-fold dilutions. All other coupled enzymes were maintained at a concentration of 1 U/ml, or 10 mU/mL for DERA. hmdUMP substrate was added to a final concentration of 100 μM, in a final well volume of 100 μL, and reacted for 3 hours at 37 °C using a SpectraMax M5 microplate reader detecting 340 nm absorbance. The assay was completed in triplicate, subtracting buffer absorbance using an intraplate control containing no NADH. Enzymatic rate is defined by the fit of initial rate data from each condition to a linear equation and the slope is divided by the extinction coefficient for NADH at e_{340} nm = 6.22 x10^{-3} μM^{-1} cm^{-1}. The observed slope is divided by 2 to correct for the dual coupled enzyme pathways, to give reaction rate units of μM dR5P produced s^{-1}.

2.4.2 pH and Temperature Optimizations—Purified DNPH1 was diluted to 30 nM in an assay mixture containing 50 mM HEPES with either pH 6.0, 7.0, or 8.0, together with 10 mU/mL (1 μM) purified DERA, 1 U/mL ADH, 100 mU/mL TPI, 100 mU/mL GPDH, and 200 μM NADH. In 96-well plates with a well volume of 100 μL, DNPH1 substrate hmdUMP was added to a final concentration of 100 μM and reacted for 3 hours at 37 °C. An identical condition to pH 7.0 was also incubated at 25°C to study the effect of

temperature on rate. As a comparison for pH and temperature effects on coupled-enzyme activity, DERA substrate dR5P was also reacted separately at a concentration of 100 μM in the same conditions. Corrected NADH absorbance and enzymatic rate were calculated as in section 2.4.1. The catalytic excess of the coupled enzyme rate was calculated by dividing the rate of the dR5P-initiated reaction by the rate of the hmdUMP-initiated reaction.

2.4.3 hmdUMP Michaelis-Menten Kinetics—Purified DNPH1 was diluted to 30 nM in an assay mixture containing 50 mM HEPES pH 7.0, 10 mU/mL (1 μM) purified DERA, 1 U/mL ADH, 100 mU/mL TPI, 100 mU/mL GPDH, and 200 μM NADH. In 96-well plates with a well volume of 100 μL, hmdUMP was added at 10 concentrations from 100 μM to 0.2 μM and reacted for 3 hours at 37 °C. Corrected NADH absorbance and enzymatic rate were calculated as in section 2.4.1. The initial reaction rates were plotted against hmdUMP concentration and fit to the Michaelis-Menten equation using GraphPad Prism to calculate K_M and V_{Max} values using the equation:

$$V = (V_{Max}[S])/(K_M + [S])$$

2.5 DNPH1 Inhibition Assay

Purified DNPH1 was diluted to 30 nM in an assay mixture containing 50 mM HEPES pH 7.0, 10 mU/mL purified DERA, 1 U/mL ADH, 100 mU/mL TPI, 100 mU/mL GPDH, and 80 μM NADH. Inhibitor concentration ranged from 1 mM to 4 μM over 9 samples. The 96-well plates with well volumes of 100 μL, were incubated for 30 minutes at 37°C and reactions initiated with 10 μM hmdUMP and monitored for 1 hour at 37 °C. Corrected NADH absorbance and enzymatic rates were calculated as in section 2.4.1. IC_{50} is calculated from the inhibited rate relative to an uninhibited control, plotted against the log of inhibitor concentration, and analyzed using GraphPad Prism fitting to the equation:

$$\text{Relative Velocity} = \text{Bottom} + ((\text{Top} - \text{Bottom})/(1 + ([I]/IC_{50})))$$

Where Top and Bottom are defined by Graphad as the maximal and minimal relative velocity values observed over the course of the titration, respectively.

K_i values were determined from IC_{50} values using the Cheng-Prusoff equation[20]:

$$K_i = (IC_{50}/(1 + ([S]/K_M))$$

Z prime (Z′) was calculated using initial rates of 340 nm absorbance loss in the first 1020 sec of assay, using three no substrate conditions for negative activity (neg), and three 10 μM hmdUMP conditions as positive activity (pos). The Z′ equation[21] is as follows:

$$Z' = 1 - ((3\,\sigma_{pos} - 3\,\sigma_{neg})/(|\,\mu_{pos} + \mu_{neg}\,|))$$

Where σ is the standard deviation and μ is the mean values from 3 conditions. A Z′ value between 0.5 and 1 is considered a quality assay.

2.5.1 DNPH1 Inhibition Assay Coupled Enzyme Activity Controls—

100 μL reaction mixtures containing 50 mM HEPES pH 7.0, 10 mU/mL (1 μM) purified DERA, 1 U/mL ADH, 100 mU/mL TPI, 100 mU/mL GPDH, and 80 μM NADH were incubated with either 1 mM AMP or GMP for 30 minutes at 37°C, then reactions were initiated with 10 μM dR5P. Reactions were allowed to proceed for 20 minutes, and initial reaction rates were compared to an intra plate control containing no inhibitor.

In a separate assay, purified DNPH1 was diluted to 30 nM in an assay mixture containing 50 mM HEPES pH 7.0, and twice the optimized coupled enzyme concentrations at 20 mU/mL (2 μM) purified DERA, 2 U/mL ADH, 200 mU/mL TPI, 200 mU/mL GPDH, and 80 μM NADH. Inhibitors AMP or GMP were added at a concentration of 100 μM. The 96-well volumes of 100 μL were incubated for 30 minutes at 37°C, reactions were initiated with 10 μM hmdUMP, and monitored for 1 hour at 37 °C. Absorbance loss at 340 nm was expressed as relative to intraplate controls containing no hmdUMP in order to account for the increase in nonenzymatic NADH oxidation from increased coupled enzyme concentrations. Rates were compared to conditions containing the standard coupled enzyme concentrations.

3. Result and discussion

3.1 Enzymatic Synthesis of hmdUMP

The modified nucleoside (hmdU) is available through commercial sources. 5′-Phosphorylation of hmdUMP through chemical reactions is hindered by the presence of primary and secondary hydroxyl groups in the nucleobase, which makes the phosphorylation process less specific and results in low yield. [22, 23] We used an enzymatic approach to selectively phosphorylate the hydroxyl group at the C5 hydroxyl of ribose (Figure 1A). This method involved the use of *Anopheles gambiae* deoxynucleoside kinase (*Ag*dNK), which has shown broad activity to produce monophosphates using unnatural nucleosides.[24] The kinase reaction uses ATP and is coupled to pyruvate kinase used to regenerate ATP. Product hmdUMP was separated from the unreacted nucleoside by anion exchange HPLC (Figure 1B) and isolated with greater than 90% yield.

3.2 Optimization of Assay Conditions

Accurate assays with coupled-enzyme systems require assurance that all coupled reactions rates are in excess of the studied enzyme. For DNPH1 activity, each of the coupled enzymes DERA, ADH, TPI, and GPDH were titrated from 1 U/mL to 51 μU/mL (10 mU/mL to 0.51 μU/mL for purified DERA), holding the other enzymes at the maximum concentration of either 1 U/mL or 10 mU/mL for DERA. DNPH1 (30 nM) was added and reactions were initiated with 100 μM hmdUMP to determine the effect of coupled enzyme concentration on the observed activity (Figure 2, A-D). The DERA titration reached the maximum observed DNPH1 activity at 3.33 mU/mL or higher. ADH showed maximum activity at or above 333 mU/mL. Both TPI and GPDH were optimal at concentrations higher than 37 mU/mL. Coupling enzyme titration experiments established the optimal concentrations for study of 30 nM DNPH1 activity to be 10 mU/mL DERA, 1 U/mL ADH, 100 mU/mL TPI, and 100 mU/mL GPDH.

Reaction pH must also be optimal for DNPH1 as well as the coupled enzymes. Assays of DNPH1 with dGMP as substrate have reported a pH optimum of 6.0.[16] We assayed DNPH1 hydrolysis of 100 μM hmdUMP in 0.05 M HEPES pH 6.0, 7.0, and 8.0. DNPH1 showed similar rates of hmdUMP hydrolysis at pH 6.0 and 7.0, with rates of 1.1×10^{-2} and 1.0×10^{-2} μM dR5P s^{-1} respectively with 50% loss of activity at pH 8.0 (SI Table 1). As a control for coupled enzyme activity with varying pH, dR5P was added for these assay conditions at 100 μM. If the coupled-enzyme activity remains in large excess, the rate of NADH oxidation will be faster than the rate of hmdUMP product formation. Coupled-enzyme consumption of added dR5P was 29-fold faster than the rate of DNPH1 at pH 7.0, and only 24-fold at pH 6.0 (SI Table 1). Further characterization of DNPH1 activity was conducted at pH 7.0, the optimal pH for DNPH1 hydrolysis of hmdUMP under these assay conditions. Additionally, DNPH1 and coupled enzyme activity were assayed similarly with a pH of 7.0 at either 25 °C or 37 °C (SI Table 2). At 25 °C hmdUMP turnover by DNPH1 was unchanged, however the coupled enzyme excess was reduced to 25-fold, leading to choice of 37 °C as the optimal reaction temperature.

3.3 Substrate Kinetic Characterization

Using 30 nM DNPH1 and the optimized conditions for assay, a titration of hmdUMP ranging from 100 μM to 200 nM was used to study the concentration dependence of the substrate on DNPH1 activity (Figure 3A). Initial rates of hydrolysis were plotted against substrate concentration and fit to the Michaelis-Menten equation to determine binding affinity, resulting in a V_{Max} of $1.0 \times 10^{-2} \pm 2 \times 10^{-4}$ μM dR5P product s^{-1} and K_M of 6.3 ± 0.5 μM (Figure 3B). The binding of hmdUMP is 9-fold tighter than the previously reported 57 μM K_M for dGMP with human DNPH1,[17] and displays a 1238-fold binding improvement over the structurally similar nucleotide dUMP with a reported K_M of 7.8 mM.[17]

The maximal rate of hmdUMP hydrolysis with 30 nM of DNPH1 represents a k_{cat} of 0.33 ± 0.007 s^{-1}, in excess of the k_{cat} value of 0.12 s^{-1} reported recently.[3] DNPH1 substrate specificity was tested with hmdUMP, dAMP, dCMP, dGMP, dIMP, dTMP, and dUMP at 100 μM with 30 nM DNPH1 (SI Figure 4). Except for slow reactivity with dUMP, rate of 0.014 s^{-1}, all other deoxynucleotides showed turnover rates < 0.01 s^{-1} (SI Figure 5). DNPH1 has a catalytic efficiency (k_{cat}/K_M with hmdUMP of 52,380 $M^{-1}s^{-1}$, making this the most efficient substrate of DNPH1 reported at this time.[3, 17] Reliable assays of DNPH1 at enzyme concentrations consistent with the detection of nanomolar inhibitors will require this robust catalytic activity of hmdUMP.

3.4 Assay of DNPH1 Inhibition by AMP and GMP

Inhibitor screens for DNPH1 are most sensitive near the K_M of hmdUMP and require a robust signal-to-noise ratio. An assay condition of 10 μM hmdUMP versus a no substrate control gave a robust Z prime value of 0.92, indicating a quality assay to measure changes in hmdUMP hydrolysis activity. Intracellular concentration of NMPs exceeds those of their corresponding dNMPs,[25] and it has been reported that some NMPs are inhibitory to DNPH1.[16, 25, 26] Inhibition tests here use the previously described inhibitors, AMP and GMP (Figure 4A).[16, 26] Relative activities of inhibited and uninhibited controls were fitted to a dose-response curve to determine IC_{50} values (Figure 4B). AMP and GMP both

displayed inhibition of hmdUMP hydrolysis with AMP titration giving an IC_{50} of 103 ± 12 μM representing a K_i of 41 ± 4.7 μM. GMP displayed a tighter binding IC_{50} at 41 ± 3.2 μM with a calculated K_i of 16 ± 1.3 μM. These inhibition profiles are similar to previous literature on the inhibition of rat DNPH1 activity with dGMP as the substrate, with K_i values for AMP and GMP of 40 μM and 20 μM respectively.[16, 26] Assay of pyrimidine NMPs (UMP and CMP) as inhibitors resulted in less than 20% inhibition at 1 mM (SI Figure 6). These data agree with the previously described weak binding affinity of similarly structured deoxy pyrimidine nucleotides.[17] As a control to study if these inhibitors act on the coupled enzyme system independently of DNPH1, 1 mM of either AMP and GMP were added to standard reaction conditions, and initiated with 10 μM dR5P. Compared to no inhibitor control, initial rates of dR5P turnover with inhibitors present were not reduced (SI Figure 7A), indicating that the observed 90% and 95% inhibition from AMP and GMP respectively at this concentration are a result of acting on DNPH1. Additionally, activity of 10 μM hmdUMP hydrolysis by DNPH1 in the presence of 100 μM of AMP or GMP was not rescued by doubling the concentration of coupled enzymes (SI Figure 7B), confirming inhibition of activity was not derived from AMP and GMP acting on the coupled enzyme system.

4. Conclusion

Robust enzymatic assays are an essential step in drug discovery screening. Common assays for enzymes in nucleotide salvage pathway often use modified purine or pyrimidines with colorimetric[27, 28] or fluorometric properties.[29] Additionally, assays can be coupled to the released nucleobase,[30] primarily for purine bases. DNPH1 has an apparent specificity for the epigenetically modified nucleotide hmdUMP,[3] and therefore measuring promiscuous activity of modified substrates is not a well-suited method to measure activity of such a specific enzyme.

Use of the natural substrate hmdUMP eliminates concerns related to substrate specificity and enzymatic rate, allowing for efficient assay of a 30 nM concentration of DNPH1. Other assays have used DNPH1 at concentrations up to 14 μM.[18] At this enzyme concentration, an inhibitor candidate with a dissociation constant of 7 μM would give less than 50% inhibition, the loss of sensitivity arising from the excess of DNPH1 relative to the inhibitors in the screen. Our assay focused on quantifying the production of dR5P and its conversion by aldolase (DERA) into metabolites, both of which can be coupled to well-known NADH-consuming enzymes allowing for improved sensitivity when substrate turnover is in the low micromolar range. The methods described here are applicable to any enzyme generating dR5P as a product.

This assay has been developed with a 96-well plate format and can be readily adapted to 384-well plates, both amenable to high throughput screening. Inhibitory data with AMP and GMP serves as proof of concept for inhibitor analysis. DNPH1 inhibitors are of current interest as anticancer agents, as they may be of use as sensitizing agents for BRCA-deficient cancers that have developed resistance to PARPi therapeutics.[3]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

Abbreviations

ADH	Alcohol dehydrogenase
***Ag*dNK**	*Anopheles gambiae* deoxynucleoside kinase
AMP	Adenosine 5′-monophosphate
dR5P	2-Deoxyribose 5-monophosphate
DERA	2-Deoxy-d-ribose-5-phosphate aldolase
DNPH1	2′-Deoxynucleoside 5′-Monophosphate N-Glycosidase 1
GMP	Guanosine 5′-monophosphate
GPDH	α-Glycerophosphate dehydrogenase
hmdUMP	5-Hydroxymethyl 2′-deoxyuridine 5′-monophosphate
TPI	Triose phosphate isomerase

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Highlights

- Chemoenzymatic synthesis of 5-hydroxymethyl 2′-deoxyuridine 5′-monophosphate from commercially available materials
- Characterization of DNPH1 activity with natural substrate 5-hydroxymethyl 2′-deoxyuridine 5′-monophosphate
- Development of an enzyme coupled assay for DNPH1 activity
- Inhibitors against DNPH1 serve as potential sensitizing agents for PARP inhibitor resistance

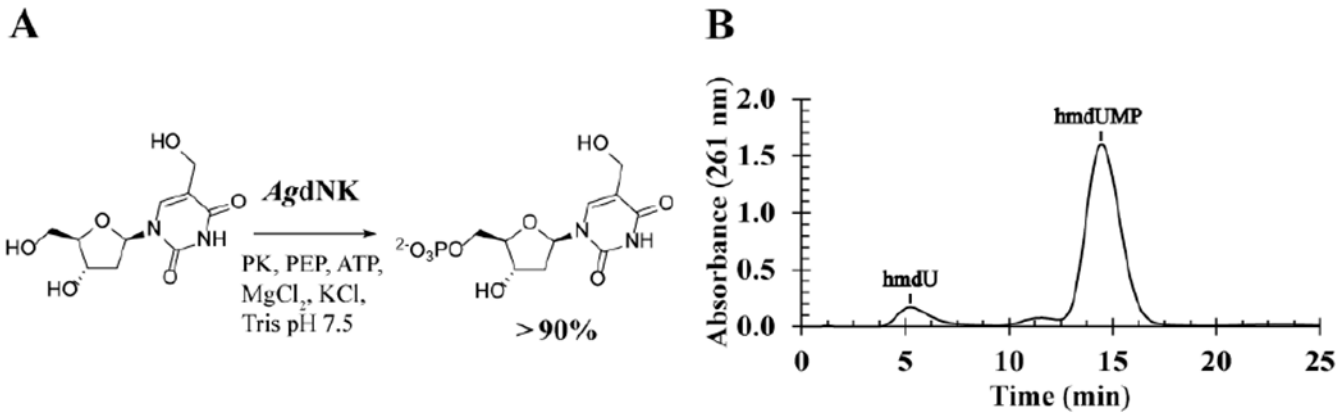


Figure 1:

Enzymatic synthesis and purification of hmdUMP. **A** – Reaction scheme using *Ag*dNK to convert hmdU to hmdUMP. **B** – HPLC elution profile for purification of hmdUMP by anion exchange chromatography.

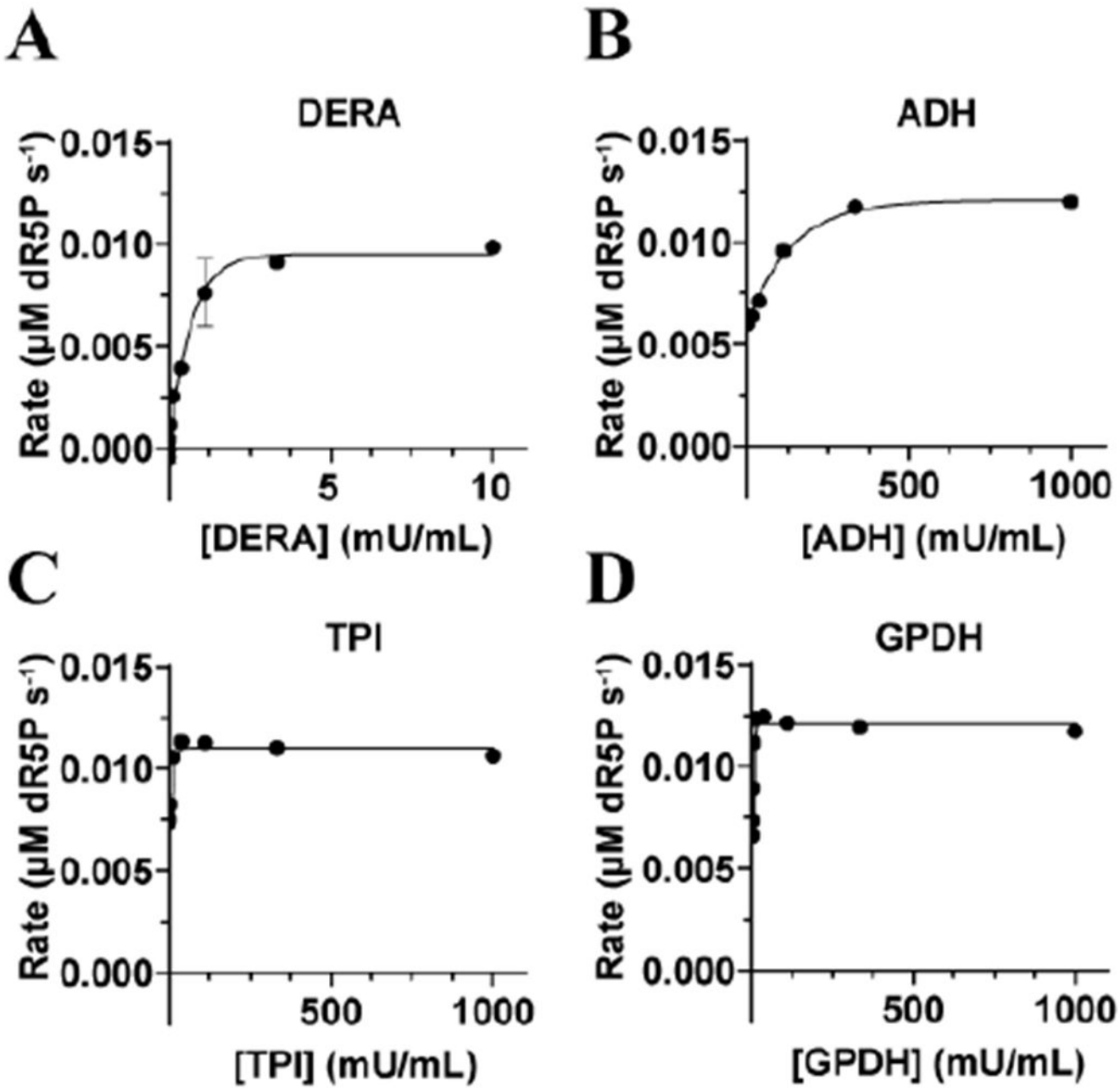


Figure 2.

Optimization of coupled enzyme concentrations, by 10-point titration of each coupling enzyme with the rest constant at 1 U/mL or 10 mU/mL for DERA. Reaction rate of 30 nM DNPH1 with 100 μM hmdUMP is plotted against titrated coupling enzyme concentrations. **A** – 2-Deoxy-d-ribose-5-phosphate aldolase, **B** – Alcohol dehydrogenase, **C** – Triose phosphate isomerase, **D** – α-Glycerophosphate dehydrogenase.

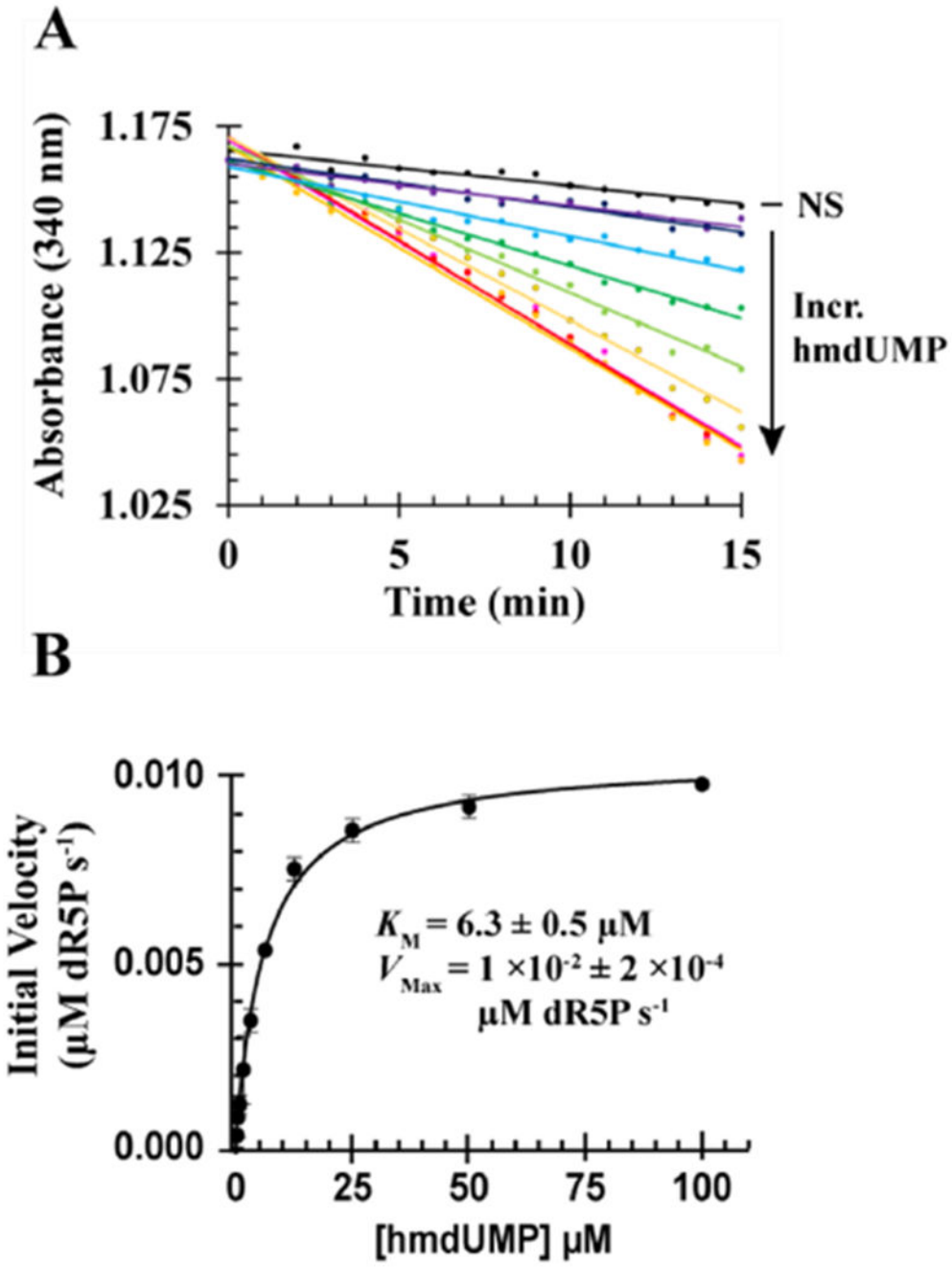


Figure 3.

DNPH1 kinetics with hmdUMP. **A** – Initial reaction rates show concentration dependent loss of NADH absorbance on titration of hmdUMP (*pink* at 100 μM to *purple* at 0.2 μM) with no substrate control (*black*). **B** – Michaelis-Menten plot of hmdUMP titration.

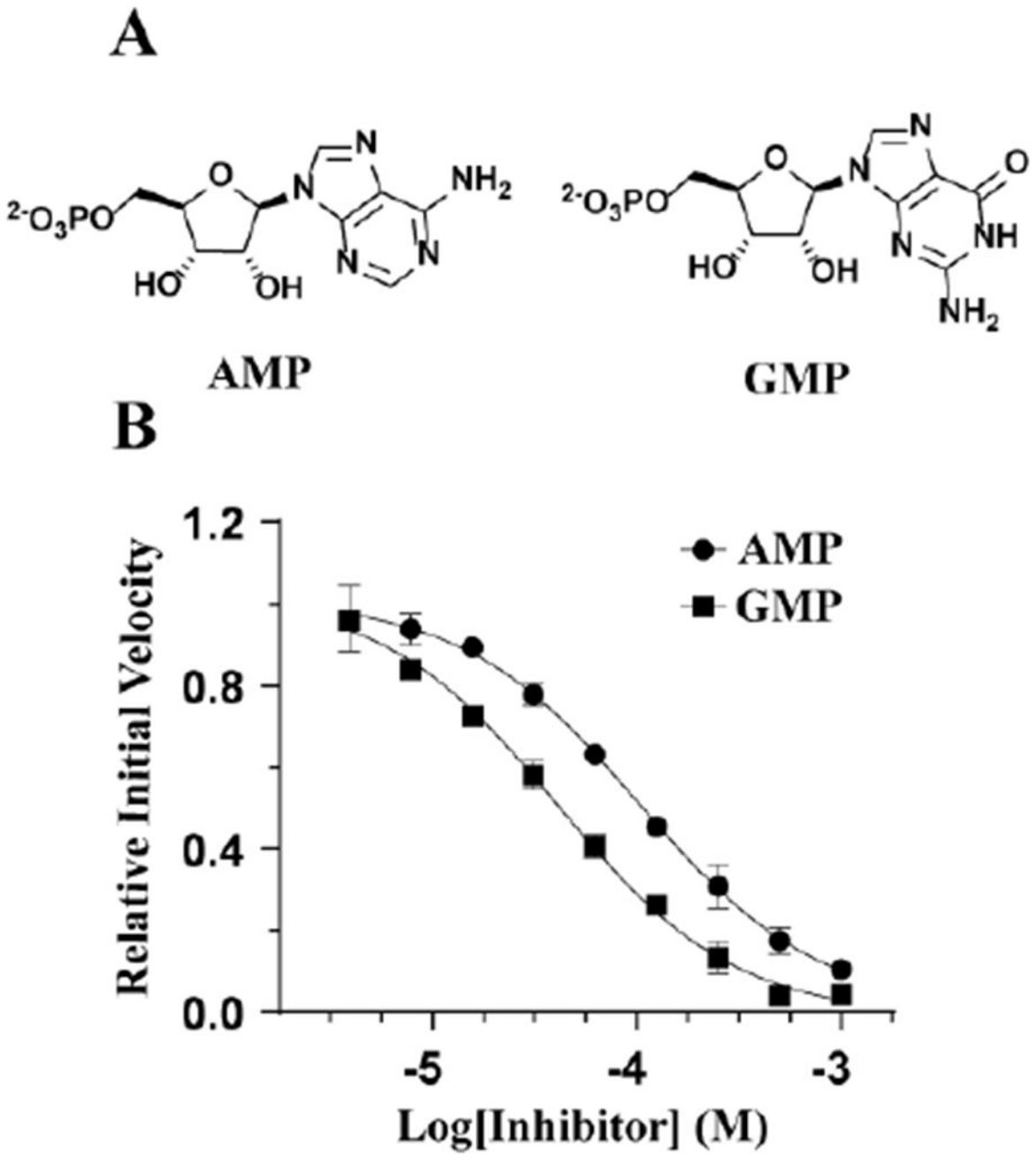
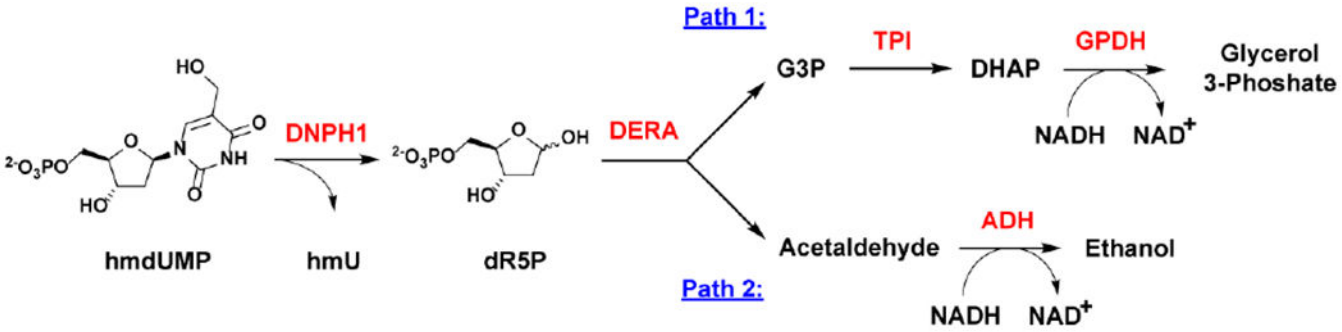


Figure 4.

Inhibition of DNPH1. **A** – Structures of AMP and GMP. **B** – Dose response curves of AMP (*circles*) and GMP (*boxes*) with 30 nM DNPH1. Initial rates relative to uninhibited controls plotted against the log of inhibitor concentration.



Scheme 1:

Dual-product enzyme-coupled assay to detect DNPH1 activity following loss of 340 nm absorbance from oxidation of NADH to NAD+.