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Communication

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A Chimeric ATP-Linked Nucleotide Enables Luminescence Signaling of Damage Surveillance by MTH1, a Cancer Target

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Supporting Information

ABSTRACT: The enzyme MTH1 cleanses the cellular nucleotide pool of oxidatively damaged 8-oxo-dGTP, preventing mutagenesis by this nucleotide. The enzyme is considered a promising therapeutic target; however, methods to measure its activity are indirect and laborious and have low sensitivity. Here we describe a novel ATP-linked chimeric nucleotide (ARGO) that enables luminescence signaling of the enzymatic reaction, greatly simplifying the measurement of MTH1 activity. We show that the reporting system can be used to identify inhibitors of MTH1, and we use it to quantify enzyme activity in eight cell lines and in colorectal tumor tissue. The ARGO reporter is likely to have considerable utility in the study of the biology of MTH1 and potentially in analyzing patient samples during clinical testing.

Cellular reactive oxygen species (ROS) can damage DNA in multiple ways.[1−3] This includes not only direct damage to the DNA itself but also to the cellular nucleotides that are used to synthesize new DNA. The most abundant forms of damage in the cellular nucleotide pool are 8-oxo-dGTP and 2-hydroxy-dATP;[4−6] 8-oxo-dGTP is particularly dangerous in the cell because DNA polymerases can mispair it with A rather than C, leading to mutations.[7,8] To ameliorate this hazard, human cells express the enzyme MTH1, an 18 kDa homologue of bacterial MutT.[9,10] Human MTH1 binds 8-oxo-dGTP and hydrolyzes its triphosphate moiety between the α and β phosphates, producing inactive 8-oxo-dGMP and pyrophosphate. Thus, the enzyme is critically important for cleansing the nucleotide pool of damage that can cause cellular mutations.

Significantly, while this activity is important for suppressing mutations in normal cells, it is not essential for cell viability.[11] However, cancer cells can be highly dependent on MTH1 to maintain their rapid growth.[12] Many tumors are driven by mutations in the *RAS* proto-oncogenes that increase reactive oxygen species, resulting in damage such as 8-oxo-dG.[13−15] Thus, RAS-dependent tumor cells often express high MTH1 levels to counteract the toxicity of elevated ROS in these rapidly growing cells.[16−19] Such studies suggest the promise of inhibiting MTH1 as a new therapeutic approach for multiple cancers.[16,20−24] Interestingly and conversely, activators of oxidative damage surveillance pathways such as MTH1 are proposed as a strategy for preventing cancers in genetically susceptible individuals.[25] As a result of these hypotheses, the MTH1 enzyme is under highly active research and debate.[22,26] Both for studying the basic science of this pathway and for development of MTH1-targeted therapies, it is critical to measure the enzyme's activity in cell lines and tumor specimens.

Despite the growing biological and clinical significance of this enzyme, current methods to measure MTH1 are laborious and indirect and have low sensitivity. Importantly, no existing method is suitable for quantifying enzyme activity in native cells or tissues. The most common in vitro methods include HPLC measurement of nucleotide cleavage to 8-oxo-dGMP[27,28] and colorimetric (malachite green) measurement of pyrophosphate,[20,29] both of which have relatively low sensitivity. Luminescence assays of pyrophosphate are more sensitive but require (in addition to MTH1 itself) both luciferase and a pyrophosphate-converting enzyme, thus adding complexity and cost.[20,21,30] Moreover, pyrophosphate detection is not specific to this enzyme pathway and has insufficient sensitivity and selectivity for use in native cells and tissues (see below). A recent study, citing the need to measure MTH1 for clinical development, reported a proteomics method for measuring protein concentration (rather than activity) involving isotope labeling, protease digestion, HPLC, and tandem mass spectrometry,[17] which because of its complexity may be difficult to develop for routine clinical use. For most applications, it would be preferable to measure MTH1 activity rather than protein quantities, since the enzyme may have variable activity due to single-nucleotide variations or post-translational modifications.[31]

Examination of the structure of MTH1 with product bound[30] shows that the terminus of the leaving pyrophosphate is likely exposed to solution, thus allowing its exit upon bond cleavage. If adenosine monophosphate were appended at this position instead (Figure 1),[32] the leaving group in this reaction would be adenosine 5′-triphosphate (ATP), which is widely employed with luciferase to generate luminescence signals. By this reasoning, we designed a two-headed dinucleotide, which we call the ATP-releasing guanine-oxidized (ARGO) probe (Figure 1). Unknowns in this design included (a) whether the AMP conjugation with 8-oxo-dGTP would hinder MTH1 activity and (b) whether luciferase might directly accept the chimeric nucleotide as a substrate, thus short-circuiting the design.

The ARGO probe was synthesized from ATP and 8-oxo-dGMP in 45% yield (see the Supporting Information (SI)).

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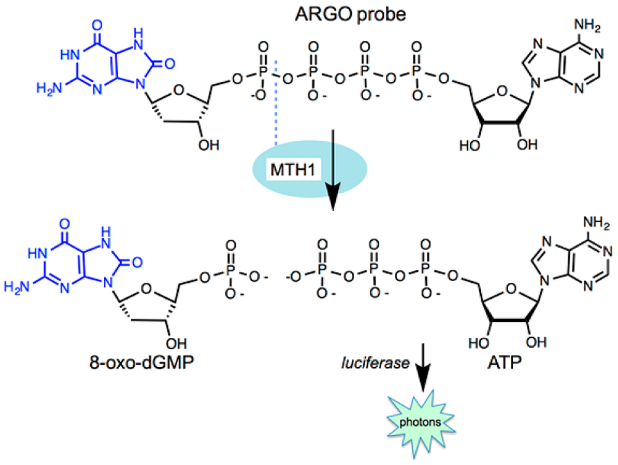
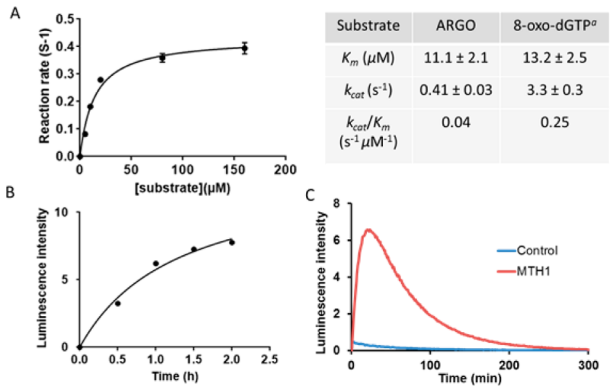


Figure 1. Chimeric ARGO probe combining 8-oxo-dGTP (damaged base in blue) and ATP. The MTH1 repair enzyme cleaves the probe, releasing ATP, which triggers luminescence signals via luciferase.

Incubation of ARGO with MTH1 cleanly consumed the compound and produced two products, which coelute with ATP and 8-oxo-dGMP by HPLC (Figure S1). Mass spectrometry confirmed these products (Figure S2). Kinetics studies of the ARGO substrate with MTH1 (Figure 2A)



Substrate	ARGO	8-oxo-dGTP[a]
K_m (μM)	11.1 ± 2.1	13.2 ± 2.5
k_{cat} (s^{-1})	0.41 ± 0.03	3.3 ± 0.3
k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	0.04	0.25

Figure 2. ARGO acts as an efficient substrate of MTH1, generating luciferase signals. (A) Michaelis–Menten fit to initial rates data with ARGO as a substrate. [a]Published values for MTH1.[30] (B) Plot of luciferase signals as a function of MTH1 reaction time with 40 μM ARGO (two-tube reaction). The data were fit to a single-exponential function. (C) Time course of signals in single-tube reaction (control = no MTH1). See the text for conditions; data in (A, B) are averages of three experiments.

revealed a K_m of 11.1 ± 2.1 μM, within experimental error of the value for the native substrate,[28,30] and a k_{cat} of 0.41 ± 0.03 s^{-1}, which is only 8-fold lower than that of the native 8-oxo-dGTP.[30] To test for background signal, we incubated the ARGO substrate with luciferase in the absence of MTH1; only very small signals were observed (Figure S3), likely from very small amounts of contaminating ATP in the synthetic nucleotide rather than from slow reaction of ARGO with luciferase.

Next we explored strategies for coupling the ARGO/MTH1 reaction with luciferase reporting. To avoid possible buffer incompatibilities, we first tested a two-tube method, performing a 100 μL MTH1 reaction with 40 μM ARGO, removing 5 μL aliquots over time, and using the aliquots to generate luciferase signals in a commercial luciferase buffer after 5 min. A plot of the data reveals signals rising over background within 5 min and reaching a plateau in ca. 120 min of MTH1 reaction time (Figure 2B). This two-tube approach offers some convenience in analyzing multiple samples accumulated over time and for cell lysate measurements (see below). However, in many applications it would be more convenient if the MTH1 activity could be measured in a single reaction. Thus, we tested a single-tube approach, adding ARGO probe and luciferase in luciferase reaction buffer with MTH1. A time plot of the luminescence (Figure 2C) shows signal increasing over an hour and then decreasing, as expected with luciferase signaling.

We evaluated the sensitivity of the ARGO reporter by varying the MTH1 concentration. A plot of the luminescence data (Figure 3A) shows a limit of detection of 0.5 nM enzyme

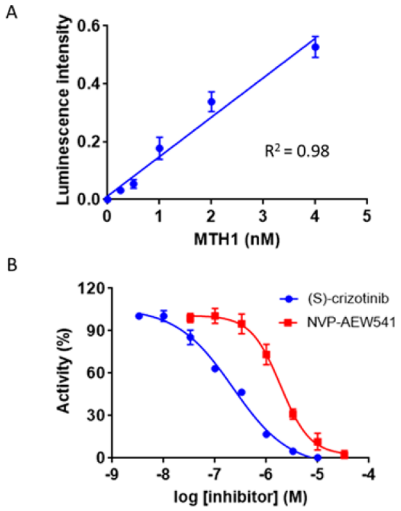


Figure 3. Use of the ARGO probe to quantify MTH1 and evaluate inhibitors. (A) Sensitivity of ARGO as measured by dilutions of the enzyme. The limit of detection is 0.5 nM enzyme using the two-tube assay. The linear fit through the data is shown. (B) Titration curves of MTH1 with two inhibitors, measured with the two-tube assay. (*S*)-Crizotinib was previously reported,[21] while NVP-AEW541 was discovered here. The log(inhibitor) vs response curves were created using fits to a variable-slope model with GraphPad.

concentration. Later experiments (below) showed that this sensitivity is sufficient to measure native levels of MTH1 in multiple tumor cell lysates.

Because of the clinical interest in targeting MTH1,[20,21,29] we confirmed the ability of the ARGO reporter to characterize small-molecule inhibitors of this enzyme. (*S*)-Crizotinib was recently reported as an MTH1 inhibitor with a reported IC_{50} of 72 nM[21] or 500 nM[29] as measured by pyrophosphate assay or malachite green assay, respectively. We performed inhibitor dilution experiments in the presence of ARGO and MTH1; plots of reaction rate versus time (Figure 3B) revealed an IC_{50} of 220 nM. To test whether the new probe could be used in library screening format to identify new inhibitors, we applied the one-tube method to evaluate a set of known kinase inhibitors, which led to the identification of NVP-AEW541,[33] previously unknown as an inhibitor of MTH1 (IC_{50} = 1.9 μM; Figure S5). Thus, the ARGO assay provides a convenient method for screening libraries for inhibitors of MTH1.

No previous MTH1 assay has been successfully employed to measure the enzyme's activity in native cell lines or tissues.

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Thus, we carried out experiments of the probe with lysates of several solid tumor cell lines. Because cells contain millimolar quantities of ATP, we used a spin filtration column to deplete lysates of ATP prior to the assay; experiments with U2OS cell lysate confirmed near-complete removal of constitutive ATP signals after washing (see Figure S6; the washing steps required only 30 min). The remaining lysate fraction was rediluted in MTH1 reaction buffer for measurement of cellular activity. A time course with 40 μM ARGO (Figure 4) revealed substantial

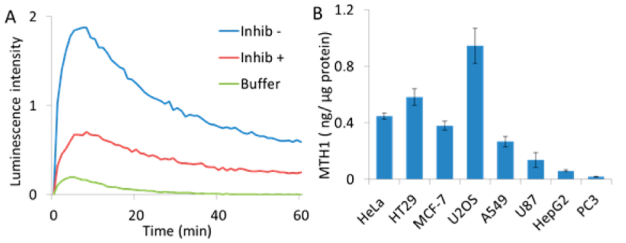

Figure 4. Employing the ARGO probe to measure MTH1 activities in cell lysates. (A) Time-course plot of luciferase signals in U2OS lysate in the absence (−) and presence (+) of the inhibitor (*S*)-crizotinib; the buffer control lacked cells. (B) Plot of MTH1 activity in various tumor cell lines. Lysates were depleted of ATP before measurement as described in the SI. Error bars are standard deviations from three measurements.

protein (Figure 5). Comparison with the matched normal colon tissue sample, which had an MTH1 level of 0.005 ±

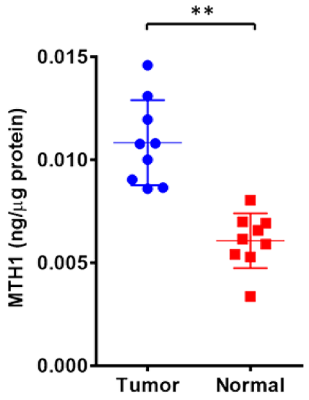

Figure 5. Use of the ARGO probe to measure MTH1 activities in colon tissue lysates. ATP-depleted tissue lysate (30 μg of total cellular protein) was added to 20 μL of MTH1 reaction buffer with 40 μM ARGO nucleotide. After 1 h of incubation at 30 °C, 5 μL of this reaction solution was added to 95 μL of luciferase reaction solution for luminescence measurement after 5 min. To calculate MTH1-specific signals, the background level with inhibitor for each lysate was subtracted from that without inhibitor (see the SI). Final measurements were averaged from nine replicates as shown (**, $p < 0.01$).

luminescence signals from 1−2 μg of cellular total protein. To extract MTH1-specific signals from the cellular background, we performed the same experiment in the presence of 20 μM MTH1 inhibitor (*S*)-crizotinib, which reduced the signals by 70%. Since our in vitro studies showed that this concentration of the inhibitor gives essentially complete MTH1 inhibition (Figure 3B), we assigned the remaining signal to background from other cellular enzyme activities. Notably, a commercial pyrophosphate luminescence assay used previously for MTH1 assays in vitro[20,21] was not able to measure signals above background for two different cell lines (Figure S7), while ARGO functioned efficiently for both.

We proceeded to quantify MTH1 activities in a variety of tumor cell lines, several of which have not been previously characterized for this enzyme. The results (Figure 4B) showed that the MTH1 activity varied widely (by a factor of 45) in the cell lines; U2OS cells have the highest levels of those measured, while PC3 cells have the lowest. Three of these cell lines (HeLa, MCF-7, and HepG2) were previously measured for MTH1 levels via mass spectrometry methods.[17] Those results reported that HeLa and MCF-7 cells have similarly high concentrations of protein, the same as the result observed here. In addition, the ratio of levels of MTH1 previously measured in HeLa versus HepG2 cells (6:1, respectively) is also very close to that observed here (7:1), providing independent confirmation of our approach. We note that our method is considerably simpler and less costly and measures activity directly rather than protein amount.

Given the ability of the ARGO reporter to quantify MTH1 activity from cultured cells, we next carried out a test of its capacity to report on the enzyme in a primary human tumor specimen. We obtained frozen *RAS* mutation-positive tumor tissue and normal colon tissue from a patient with colorectal cancer and prepared ATP-depleted lysates from it (see the SI for details). We then used the ARGO reporter to measure a tumor MTH1 activity of 0.011 ± 0.003 ng/μg of cellular

0.003 ng/μg, established a 2.2-fold elevated signal in the tumor ($p < 0.01$). These values fall within the concentration range reported recently for tumor specimens as measured by the prior proteomics/mass spectrometry method.[17] Thus, we conclude that it is possible to use the ARGO probe methodology to quantify MTH1 activity in clinical cancer samples by relatively simple luminescence measurements.

In summary, we have shown that the ARGO probe design functions effectively to signal MTH1 activity in vitro and in cell and tissue lysates. The new method is rapid and simple and can be used to quantitate activity directly rather than indirect metrics such as mRNA or protein amount. Compared with the common malachite green pyrophosphate assay (detection limit of ∼0.5 nmol of pyrophosphate),[20,29] the sensitivity of ARGO is greater as a result of the high sensitivity of luciferase for ATP (detection limit of ∼0.5 pmol of ATP). Moreover, pyrophosphate assays are not selective for this enzyme and have not been useful for quantitative measurement of native enzyme from cells, while the current method is the only existing approach that has been shown to measure MTH1 activity from native cells. The enzymatic efficiency is relatively high for the ARGO probe; while the modestly reduced k_{cat} value slows the generation of signals relative to the theoretical maximum of the native substrate, we still observe maximal signals in conveniently short amounts of time (30 min). More importantly, the K_m value of the ARGO substrate is identical to that of the native damaged nucleotide.

If MTH1-targeted therapies proceed to clinical stages, the ARGO probe may enable the quantitative selection of patients for such targeted therapy by analysis of biopsy specimens. Since the probe measures activity directly rather than protein quantity, it should be able to report on differential activity of mutants as well.

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■ ASSOCIATED CONTENT

Supporting Information

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Experimental procedures; synthesis and characterization data; screening and analytical data (PDF)

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Notes

The authors declare no competing financial interest.

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