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Direct Measurement of the Intracellular Concentration of 8-Oxo-2'-Deoxyguanosine-5'-Triphosphate by LC-MS/MS

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Abstract

Exposure to reactive oxygen species can result in formation of oxidized nucleotides which increase the frequency of mutations, DNA damage, and cell death. MutT Homolog 1 (MTH1) is an enzyme that catalyzes removal of pyrophosphate from the highly mutagenic oxidized nucleoside triphosphate 8-oxo-2'-deoxyguanosine-5'-triphosphate (8-oxo-dGTP). Interest in MTH1 as a potential new target for cancer therapy surged due to reports of MTH1 inhibitors causing DNA damage and inducing cell death in cancer cells. However, questions have been raised about MTH1 target validation. One critical piece of information that is currently lacking is a quantitative understanding of the levels of 8-oxo-dGTP in cancer cells and the effect of MTH1 inhibition on them. In this study, we developed a sensitive and selective method to simultaneously measure the intracellular concentrations of 8-oxo guanosine nucleotides and their unmodified counterparts using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). An ion-pairing reversed phase liquid chromatography method using dimethylhexylamine was employed to quantify the highly polar analytes. The method was validated fit for purpose using a combination of authentic standards and cell samples. The intracellular oxo-nucleotide concentrations in human bone osteosarcoma cell line U2OS were determined using the method. We observed very low levels of 8-oxo nucleotides in untreated and hydrogen peroxide treated cells and MTH1 knockdown in these cells had minimal if any effect on 8-oxo nucleotide levels. This method will allow for a more comprehensive understanding of oxidized nucleotide detoxification pathways and MTH1 as a target for cancer therapy.

Keywords: 8-Oxo-dGTP • LC-MS/MS • Reverse phased chromatography • Ion pairing • MutT homolog 1 (MTH1).

Introduction

Modification of nucleic acids can occur by exposure to reactive oxygen species (ROS) derived from oxidative stress, chemicals, and environmental elements [1,2]. Oxidation of nucleobases can occur in DNA or RNA strands as well as at the nucleotide levels [3]. Modified nucleotides cause mutations that can contribute to the development of cancer. Furthermore, high levels of oxidized nucleotides in cellular nucleotide pools increase the occurrence of oxidized bases, abasic sites and strand breaks in DNA which can result in cell death [4]. One important modification is the oxidation of guanosine to form 8-oxoguanosine. The 5'-triphosphate form of 8-oxo-2'-deoxyguanosine (8-oxo-dGTP) can be efficiently incorporated into a DNA strand opposite dC and dA by various mammalian replicative and repair DNA polymerases [5]. When 8-oxo-dG is in the template opposite the incoming nucleotide, the incorrect dA can be efficiently inserted which further increases the mutation rate [6-8].

There are defense mechanisms to mitigate oxidized nucleotide-mediated DNA damage and cell death. MTH1 (MutT homolog 1) is an enzyme that catalyzes the hydrolytic removal of the pyrophosphate of oxidized purine nucleoside 5'-triphosphates to form the corresponding monophosphate that can no longer be incorporated into elongating DNA. In bacteria MutT was identified to cause a 100-fold to 10,000-fold increase in the A-T/G-C trans version rate [9,10]. Therefore, inhibition of MTH1 has been pursued as a possible means to promote cell death in cancer cells and a number of potent inhibitors of the MTH1 enzyme have been identified [11-13]. Several cellular assays monitoring the DNA damage response, anti proliferative activity, and effects of MTH1 knockdown have been reported [14,15].

In order to determine the role of MTH1 in modulating levels of oxo-NTPs, it is important to have a quantitative understanding of the intracellular concentrations of 8-oxo-dGTP and the effects of MTH1 inhibition in eukaryotic cells. Herein, we report the development and partial validation of a sensitive and specific method to quantify intracellular 8-oxo nucleotide levels in the human bone osteosarcoma cell line U2OS using LC-MS/MS. The analytical method was suitable for the accurate quantitation of the intracellular levels of 8-oxo-dGTP and 8-oxo-GTP relative to the respective unoxidized nucleotides dGTP and GTP. More generally, the described methods will aid in the assessment of *in vitro* and *in vivo* levels of 8-oxo-guanosine nucleotides and the further study of the role of MTH1 and perhaps other nucleotide detoxifying enzymes in controlling their levels. The application of these methods in the characterization of novel and potent inhibitors of MTH1 was recently reported [13].

Materials and Methods

Reagents

8-Oxo-2'-deoxyguanosine-5'-triphosphate (CAS #139307-94-1; molecular weight 523.1 g/mol) was purchased from Tri Link Biotechnologies (San Diego, CA). 8-Oxo-guanosine-5'-triphosphate (CAS #21238-36-8; molecular weight 539.2 g/mole) was purchased from Jena Biosciences (Jena, Germany). 2'-deoxyguanosine-5'-triphosphate (CAS #18423-40-0; molecular weight 507.2 g/mol) and guanosine-5'-triphosphate (CAS # 36051-31-7; molecular weight 523.2 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO). Stable isotope labeled 8-Oxo-guanosine-5'-triphosphate (molecular weight 542.1 g/mole) was purchased from Toronto Research Chemical. Stable isotope labeled 2'-deoxyguanosine-5'-triphosphate (molecular weight 522.1 g/mol) and guanosine-5'-triphosphate (molecular weight 538.1 g/mol) were purchased from Sigma-Aldrich. A 100 µM stock solution in deionized water was prepared for each analyte and was made for further dilution to make working standards for LC-MS/MS analyses. The stock solutions were stored at -80°C when not in use.

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Cells

U2OS cells (osteosarcoma cell line) were purchased from the American Type Culture Collection (Manassas, VA) and shRNA (short hairpin ribonucleic acid) – mediated knock down was performed as described previously [13]. Cells were cultured in T175 Vented Flask (Corning, Kennebunk, ME) in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) with 10% fetal bovine serum (FBS; Sigma-Aldrich) and penicillin-streptomycin (Irvine Scientific, Santa Ana, CA). A final concentration of 400 µg/mL of G418 (Sigma-Aldrich) was supplemented in shRNA knockdown cells to maintain the selection. Approximately 10 million cells were cultured in each T175 flask.

Sample preparation

U2OS cells were treated with MTH1 inhibitor for 48 h before harvesting [13]. Following removal of extracellular media, control or treated cells were trypsinized and combined into a 15 mL conical tube, and then washed twice with 4 mL of ice-cold 0.9% normal saline. The cell pellets were quenched with 1 mL ice-cold 70% methanol containing 500 nM 2-chloro-adenosine-5'-triphosphate (Sigma-Aldrich) as an internal standard. Samples were stored overnight at -20°C to facilitate nucleotide extraction.

Cellular debris from samples were removed by centrifuging at 15,000 x g for 15 min and transferring the supernatant to a clean tube. The supernatant was dried on a MiVac Duo concentrator (Genevac, Gardiner, NY). Dried samples were then reconstituted in 40 µL of 1 mM ammonium phosphate buffer (pH 7.4) for LC-MS/MS analysis with an injection volume of 10 µL on the column. Sample stability in the LC-MS/MS injection buffer was found to be greater than 24 h at 4ºC based on multiple injections of the same sample from an auto sampler cool stack.

Hydrogen peroxide treatment

In the hydrogen peroxide treatment experiment, U2OS cells were incubated at 37ºC with varying concentrations of hydrogen peroxide (Sigma-Aldrich) for 90 min. The concentrations of hydrogen peroxide were in the range of 0.01 mM-10 mM. Reactive oxygen species were measured using CellROX Green according to the manufacturer's protocol (Thermo Fisher). In a separate experiment, approximately 10 million U2OS cells were incubated with 1 mM hydrogen peroxide (10% in aqueous solution) at 37°C for intracellular oxo-NTP measurement. After 90 min incubation, cells were washed with 10 mL of ice-cold 0.9% normal saline and the cell pellets were quenched with 1 mL ice-cold 70% methanol.

Liquid chromatography

Analytes were separated on a 50 x 2 mm x 2.5 µ Luna C18 (2) HST column (Phenomenex, Torrance, CA) using an ion pairing buffer containing 3 mM ammonium formate (pH 5) and 10 mM dimethylhexylamine (DMHA; Sigma-Aldrich). Mobile phase A contained 3 mM ammonium formate and 10 mM DMHA in 100% water, and mobile phase B contained 3 mM ammonium formate and 10 mM DMHA in 50% acetonitrile. Mobile phases were filtered through an Altech (Deerfield, IL, USA) vacuum filter flask apparatus using a nylon 0.2 µm filter. All other chemicals were the highest grade available from Sigma-Aldrich. A linear gradient was applied for the analysis with a flow rate of 150 µL/min from 80% mobile phase A for the first 1.5 min to 1% mobile phase A over 8 min and then followed by 5 min re-equilibration to 80% mobile phase A. Flow was directed from the column to the mass spectrometer via divert valve prior to analyte elution and directed back to waste post the analyte solution. Syringe wash solutions were prepared in 1% formic acid in water and 1% formic acid in 50% acetonitrile. The syringe and injection port were washed 2 times with the aqueous and acetonitrile wash solutions after each injection.

Instrumentation

Samples were injected on a CTC Analytics LEAP auto sampler (Leap Technologies, Carrboro, NC, USA). A Shimadzu LC-20AD (Shimadzu Scientific Instruments, Columbia, MD, USA) tertiary high-performance liquid chromatography system was used to maintain 150 µL/min flow rate and mediate the gradient. Peek tubing (Western Analytical, Boise, ID) with 0.062 µm × 125 µm internal diameter was used throughout allowing for a small dead volume. A 6-port Valco valve (Valco Instruments Co., Inc., Houston, TX) was used to direct flow to either the mass spectrometer or to waste. The third pump was attached separately to the valve giving a constant flow of 200 µL/min 50% acetonitrile to the mass spectrometer source when the LC flow was diverted to waste in order to facilitate cleaning. The LC system was connected to a Sciex API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) running in positive ion multiple reaction monitoring (MRM) mode with the general parameters listed in Table 1 and analyte specific parameters listed in Table 2.

Quantitation methods

For quantitation, 7 points standard curves were made in untreated cell matrices covering 3 orders of magnitude of analyte concentration. The analytes concentrations were prepared in the range of 8.2 nM to 2000 nM (0.082 pmols to 20 pmols on column) and normalized to pmol/million cells based on the cell counts from individual experiments. For 8-oxo-dGTP and 8-oxo-GTP, the calibration curves were in the range of 0.003 pmol/million cells to 0.6 pmol/million cells. For dGTP and GTP, the calibration curves were in the range of 0.33 pmol/million to 80 pmol/million cells. To avoid the carry over and allow for optimal lower limits of quantitation three blank injects were used following calibration standards and quality control samples. In all cases, linearity exceeded an R^2 value of 0.98 - 0.99. To assure precision within 20% over the course of the analysis, standard curve samples were injected at the beginning and end of the sample set.

Results

LC-MS/MS method development

MTH1 is a pyrophosphatase which selectively recognizes oxidized

Table 1. Summary of general MS parameters.

Probe height	2 mm
Curtain gas (CUR)	30 psi
Collision gas (CAD)	9 psi
Ion spray voltage (IS)	5500 V
Temperature	550ºC
Polarity	Positive
Ion source gas 1 (GS1)	40 psi
Ion source gas 2 (GS2)	40 psi
Resolution Q1	Unit
Resolution Q3	Unit
MR pause between scans	5ms
Dwell time	100 ms
Entrance potential	10V

Table 2. Summary of analyte specific MS parameters.

Analyte Parameter	Mass Transition [m/z]	Declustering Potential (DP) [V]	Collision Energy (CE) [V]	Collision Cell Exit Potential (CXP) [V]
GTP (Stable isotope)	524.2 / 152.1 (539.2 / 162.1)	239 (237)	35 (26)	18 (22)
dGTP (Stable isotope)	508.1 / 152.2 (523.2 / 162.1)	292 (237)	30 (26)	21 (22)
8-oxo-GTP (Stable isotope)	540.3 / 168.2 (543.1 / 171.0)	288 (288)	41 (45)	17 (22)
8-oxo-dGTP	523.8 / 168.2	288	41	17

purine nucleoside triphosphates, thus converting 8-oxo-dGTP or 2-OH-dATP to 8-oxo-dGMP or 2-OH-dAMP, respectively [16].The structures and molecular weights of natural or oxidized purine nucleotides are summarized in Figure 1. Developing the LC-MS/MS method for 8-oxo-dGTP and 2-OH-dATP was attempted. While the m/z value for 8-oxo-dGTP is the same as GTP and they elute at the same retention time on liquid chromatography, they can be separated by triple quadrupole mass spectrometry because of their different daughter ions (nucleobases). While a similar mass spectrometry MRM approach was used for the detection of 2-OH-dATP, its parent and daughter ions possess the same m/z values as endogenous dGTP and ATP and development of a reliable analytical method of intracellular 2-OH-dATP is technically challenging and requires further method development and optimization to avoid the interference in MRM channel of 2-OH-dATP **(**Supplemental Figure 1 **).** Therefore, an ion-pairing LC-MS/MS method to measure the intracellular levels of 8-oxo-dGTP and 8-oxo-GTP in U2OS cells is described below. U2OS cells have been used in biological assays for MTH1 activity and inhibition [14].

The mass spectrometer was tuned for 8-oxo-dGTP, 8-oxo-GTP, dGTP, and GTP. Product ion scans were generated in positive ionization mode for each analyte and a unique product ion was formed as the major fragment for 8-oxo-dGTP, 8-oxo-GTP, dGTP and GTP (Figure 2). The generation of unique fragments is an advantage of analyte separation using triple quadrupole mass spectrometry. Especially since 8-oxo-dGTP and GTP share the same mono-isotope (M+H) on mass spectra the cross-talk effect was also evaluated to confirm the selectivity for each analyte (Figure 3). The analytes were also tuned on negative mode but the ionization efficiency is much stronger on the positive ion mode for guanosine based nucleotides. We chose to pursue the positive mode MS/MS detection method for endogenous guanosine nucleotides.

The negatively charged phosphate groups of nucleotides are very polar and hard to be retained using typical reversed phase liquid chromatography. A mobile phase containing ion-pairing agent, dimethylhexylamine (DMHA), was used to facilitate retention on a hydrophobic column and allowed for the analysis of endogenous guanosine nucleotides on a single chromatographic injection. The extensive equilibration is needed for ion-pairing, so the total run time is approximately 14 min. The mobile phase has relatively high pH with the ion-pairing agent. The pH is approximately 10 and this made column selection very challenging. As reported previously [17,18], various

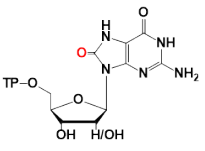
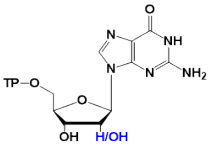
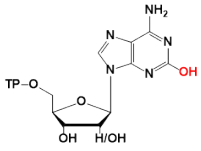
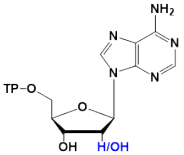
	8-oxo-dGTP / GTP	dGTP / GTP	2-OH-dATP / ATP	dATP / ATP
				
Molecular weight	523.2 / 539.2	507.2 / 523.2	507.2 / 523.2	491.2 / 507.2

Figure 1. Summary of the structures of oxidized nucleotides and corresponding molecular weights.

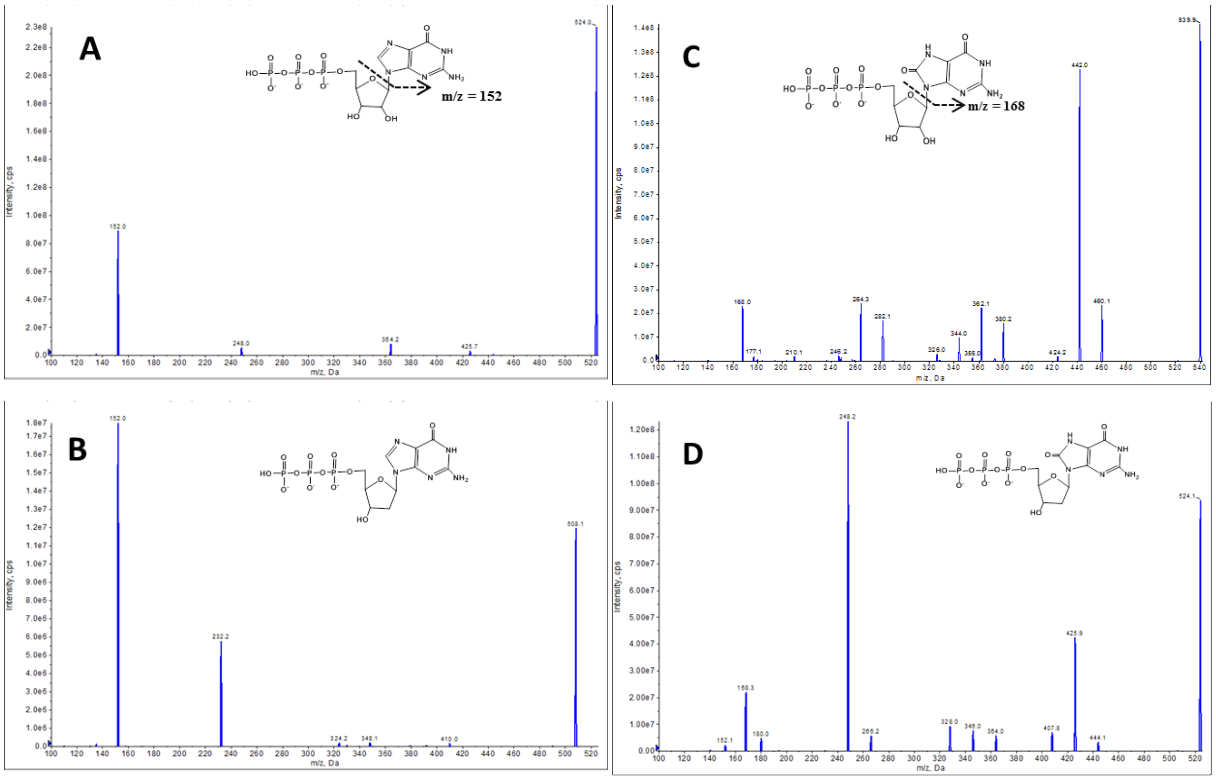


Figure 2. Product ion spectra obtained from infusions of 0.5 µM of analyte in 50% acetonitrile and 10 mM DMHA monitoring with positive ionization mode using multiple cycle averaging (MCA).
A, B) Production spectra of GTP and dGTP (m/z =524 and 508). Following optimization, the most abundant fragment observed was guanine base (m/z = 152).
C,D) Product ion spectra of 8-oxo-GTP and 8-oxo-dGTP (m/z =540 and 524). Following optimization, the most abundant fragment observed was 8-oxoguanine base (m/z = 168).

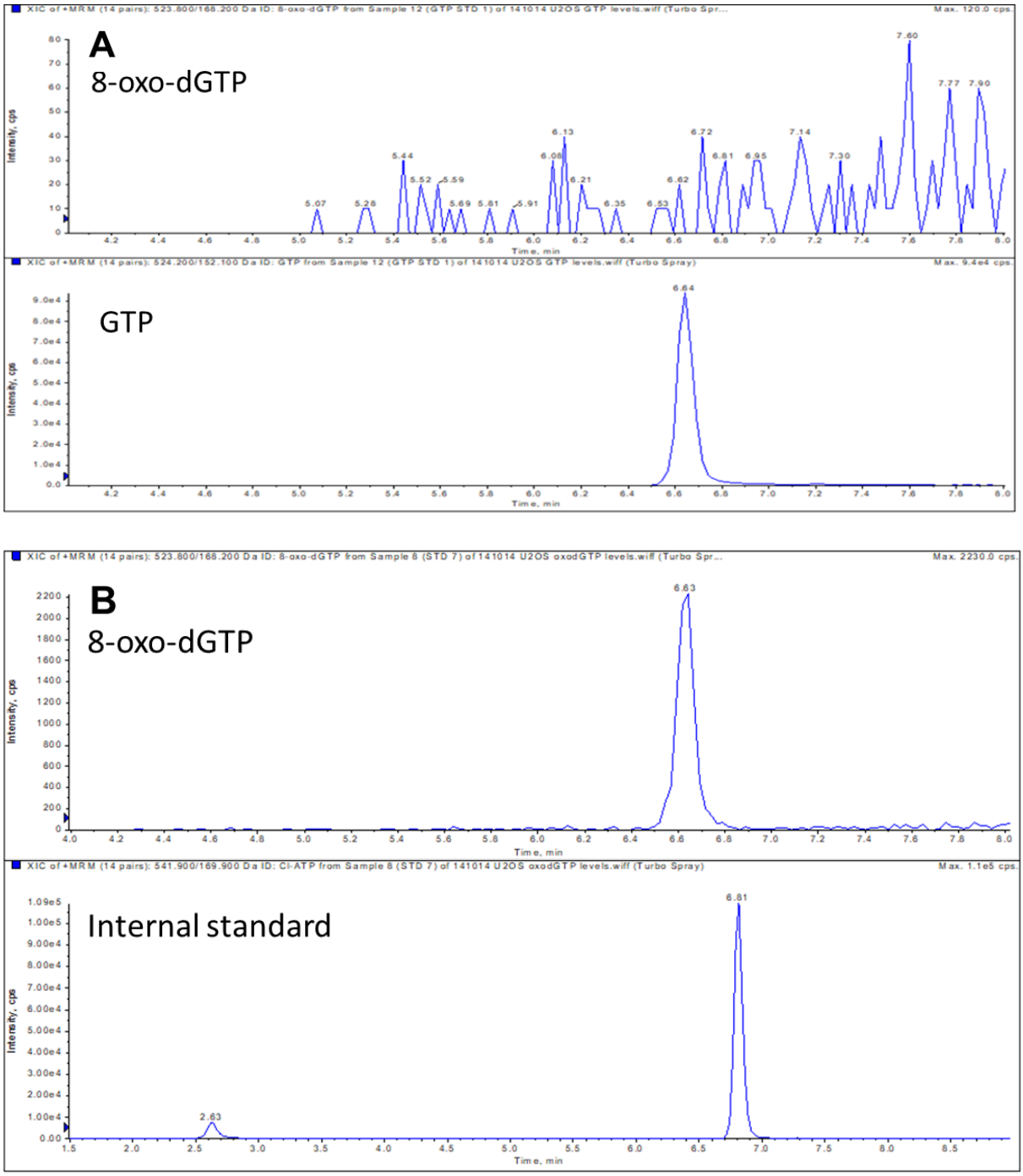


Figure 3. A) Chromatographic selectivity of 8-oxo-dGTP and GTP. Unsmoothed chromatograms from an injection of 200 nM (2 pmol on column) of neat solution of GTP, no detected 8-oxo-dGTP at the same retention time. B) The chromatograms of 8-oxo-dGTP standard at the concentration of 0.9 pmol/million cells.

C18 columns including the X terra, YMC, Acquity and Luna were tested under the similar condition. Noticeably longer column life was observed from Acquity and Luna columns, with greater than 500 injections compare to numerous other columns. With further considerations of chromatogram peak shape, pump back-pressure, column equilibrium time, and cost, the Luna column was finally chosen to pursue the liquid chromatography.

Intracellular levels of oxidized nucleotides

The method allowed the detection of 8-oxo-dGTP, 8-oxo-GTP, dGTP and GTP in cellular matrices. Cell lyses and extraction were conducted in 70% methanol at -20°C as is typically used for the measurement of endogenous nucleotides [13]. In order to avoid precipitation of endogenous nucleotides, the level of organic solvent was limited. The method was validated fit for purpose for use in measuring *in vitro* samples. Triplicate injections of the same sample of 1 million cells extract from untreated U2OS cells resulted in coefficient of variation (CV) not greater than 20% for each analyte. Comparison of levels from the three independent experiments resulted in CV values of 7.97, 16.1, 11.5 and 8.98% for 8-oxo-dGTP, 8-oxo-GTP, dGTP, and GTP, respectively. Carry over observed within the chromatographic regions of all the analytes and internal standard was < 20%. Furthermore, the intracellular levels of GTP and dGTP determined following this extraction procedure were consistent with the levels determined using different extraction and detection techniques in the previous report, indicating that this extraction method is effective and sufficient [19,20]. While a formal freeze thaw validation was not conducted, no degradation of the nucleotides was evident in the samples stored at -20°C in 70% methanol for 72 h versus the samples from the same experiment without the storage in freezer. Calibration standard curves were constructed in cell matrices and covering the concentration range in excess of 3-orders of magnitude. The experiment started with 1 million cells based on the previous works, but the 8-oxo-dGTP

was undetectable. The cell counts were then increased to 10 million cells, and then further increased to 30 million cells. While the basal level of 8-oxo-dGTP in U2OS cells was very low, a distinct peak of 8-oxo-dGTP with signal-to-noise ratio greater than 3-fold was observed in the sample prepared from 30 million cells (Figure 4). Because of the low concentration of 8-oxo-dGTP in cells, the calibration standard curve was constructed in 1 million cells and the concentration of lower limit quantitation was extra polated to 30 million cells assuming similar matrix effect in response of analytes and internal standard. Lower limits of quantitation, defined as allowing for acceptable accuracy and precision, and generally having peak heights 3-fold greater than background, were observed to be between 0.003 pmol/million cells – 0.1 pmol/million cells for each analyte. In the previously published articles, 8-oxo-dGTP was estimated to be approximately 1% – 10% of dGTP in mitochondria. The 8-oxo-dGTP level in U2OS cells was determined to be 0.008 pmol/million cells (<0.1% of dGTP) which is estimated to be 2 nM based on the cell volume of U2OS cell of 4 pL [21] (Table 3). In order to assess the effect of MTH1, the intracellular levels of 8-oxo-dGTP were also determined in U2OS cells treated with either a control shRNA or MTH1 knockdown shRNA to be 0.006 pmol/million or 0.010 pmol/million cells, respectively (Table 3). A greater than 50% knockdown of MTH1 with the specific shRNA was confirmed by Western blot analysis (data not shown). In addition, 8-oxo-GTP, dGTP and GTP levels were also measured in each cell preparation (Table 3). Due to the higher basal intracellular levels of endogenous 8-oxo-GTP, dGTP and GTP, the intracellular concentrations were determined by using the calibration standard curve generated from the corresponding stable labeled isotope standard. Results indicated that the transient knockdown of MTH1 did not alter the intracellular concentrations of the oxidized nucleotides. The values for dGTP and GTP were in the normal range of reported literature values [19,21].

Effects of H_2O_2

We then treated the cells with hydrogen peroxide which is known to increase the reactive oxygen species (ROS) [22]. First, the U2OS cells were treated with H_2O_2 for 90 min at different concentrations (0 mM-10 mM) and levels of ROS were measured using CellROX Green, a green fluorogenic probe which shows bright green fluorescence upon oxidation by ROS. The highest signal was observed at 1.11 mM of H_2O_2 and at higher concentrations, the signal declined due to cell death (Figure 5A). Therefore, 10 million U2OS cells were treated with 1 mM H_2O_2 for 90 min to facilitate oxidation of endogenous nucleotides. Since lower cell numbers were used in this experiment, 8-oxo-dGTP was not measurable either with or without H_2O_2 treatment (<0.02 pmol/million cells). Increased ROS levels are expected to elevate both 8-oxo-dGTP and 8-oxo-GTP levels in the nucleotide pool, therefore, 8-oxo-GTP was used as a surrogate for guanine

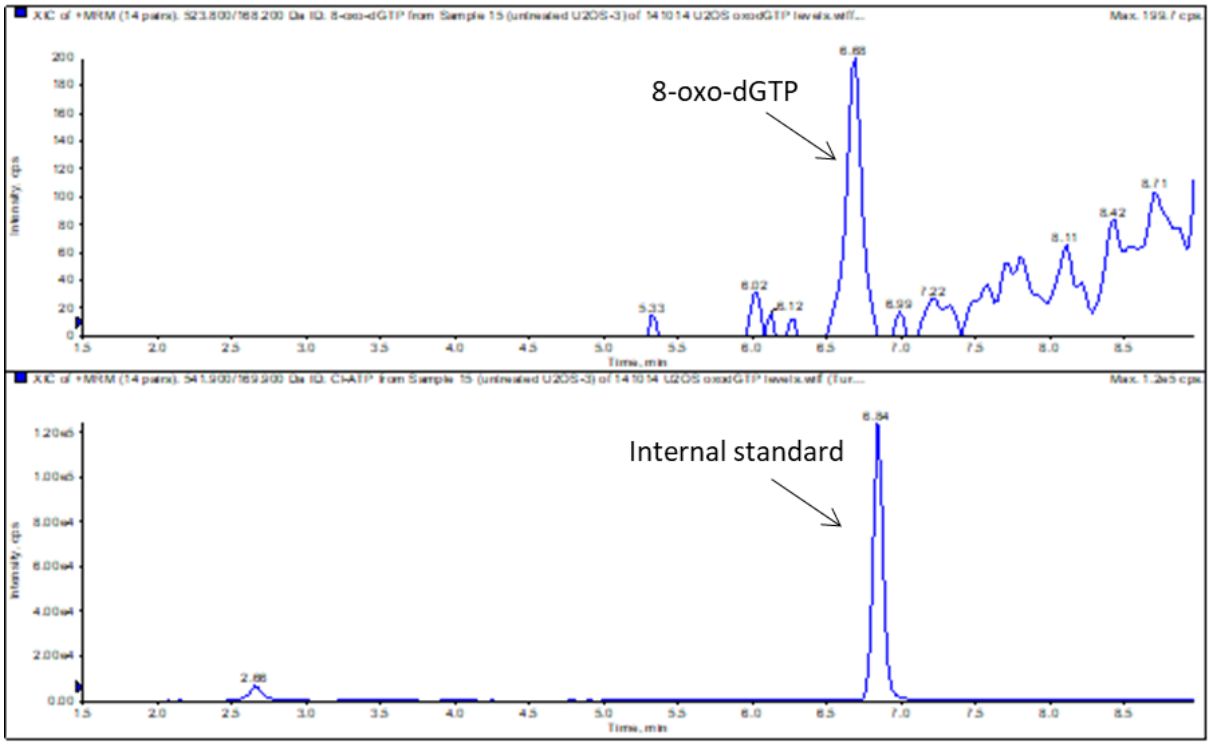


Figure 4. Extremely low concentration of 8-oxo-dGTP was measured in cells. Unsmoothed chromatogram from an injection of untreated U2OS cells.

Table 3. Intracellular concentrations of oxo-NTPs in untreated, control shRNA, and MTH1 shRNA treated U2OS cells[a].

Treatment[b]	Intracellular Concentration (pmol/million cells)							
	8-oxo-dGTP[c]		8-oxo-GTP		dGTP		rGTP	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
No Treatment	0.008	< 0.003	0.058	0.038	14.9	19.1	4,370	ND[d]
Control shRNA[e]	0.006	0.005	0.034	0.026	7.43	8.64	ND	ND
MTH1 shRNA[f]	0.01	0.006	0.021	0.018	5.58	6.53	ND	ND

Results represent two independent measurements:
[a] Partial data has been previously reported in ACS Med Chem Lett 2019;
[b] 30 million cells were used for each experiment;
[c] LOQ = 0.004/0.003 pmol/millions cells from n=2 experiments;
[d] ND: Not determined;
[e] non-targeting (NT); shRNA, short hairpin ribonucleic acid;
[f] ~50% knockdown when harvested.

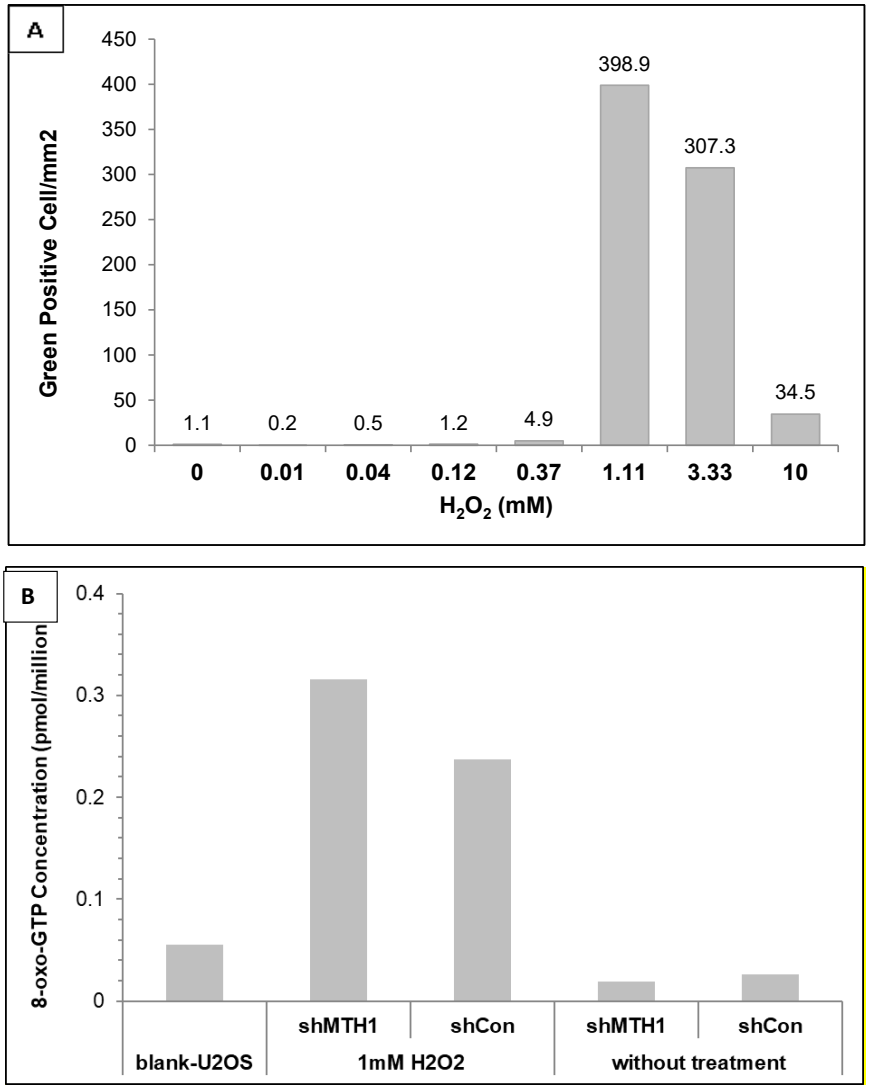


Figure 5. A) Intracellular ROS measured by Cell ROX Green signal in U2OS cells with concentration dependent incubation of H_2O_2 at 37ºC. The signal started declining at 1.11 mM of H_2O_2 treatment indicating cell death.
B) Intracellular concentration of 8-oxo-GTP in U2OS cells following the incubation of 1 mM H_2O_2 at 37ºC.

oxidation. Approximately 10 fold or 17-fold increase in 8-oxo-GTP levels was observed upon H_2O_2 treatment in control shRNA-treated or MTH1 knockdown cells, respectively (Figure 5B). The MTH1 knockdown resulted in a slight (approximately 25%) increase in 8-oxo-GTP levels with H_2O_2 treatment relative to control cells.

Discussion

While there has been enthusiasm for pursuing MTH1 inhibitors for cancer, the target has remained controversial [11,13,23,24]. 8-oxo-dGTP is a physiologically important carcinogen and loss of MutT in bacteria leads to large increases in mutations, however, the intracellular levels of 8-oxo-dGTP have not been determined in eukaryotic cells. Our observation of low levels of 8-oxo nucleotides and little to no effect of the MTH1 inhibitors may be consistent with the presence of redundant detoxification pathways in eukaryotic cells. Consistent with this hypothesis, Samaranayake et al. observed no effect on 8-oxo-dGTPase activity of 5 published MTH1 inhibitors or genetic knockdown of MTH1 [25].

Previously Tassotto and Mathews attempted to measure the intracellular levels of 8-oxo-dGTP in E. coli using HPLC with electrochemical detection and determined the levels to be below limit of detection, with a lower limit of detection of 6 pmol [26]. More recently, Fuchi, Y.et al. developed a time-resolved fluorescence assay using a 8-oxo-dGTP specific luminescent europium sensor, however, the detection method has not been applied to measure the intracellular levels of 8-oxo-dGTP [27]. Pursell, Z. F. et. al. estimated rat intramitochondrial levels of 8-oxo-dGTP to be 0.2-1.7 μM which are 10-fold to 100-fold lower than those of dGTP using a HPLC with dual channel of UV absorbance and electrochemical detection [20]. However, high signal-to-noise level evident in the published chromatograms likely adversely affected precise quantification.

In this study, we report a LC-MS/MS method with ion-pairing agent to measure the intracellular concentrations of 8-oxo-dGTP and 8-oxo-GTP *in vitro* that exhibited excellent sensitivity, with the lower limit of quantification of 0.004 pmol/million cells, and high specificity imparted by using MS/MS detection. Even using this highly sensitive method, it was challenging to obtain the distinct 8-oxo-dGTP peak and a large number of cells were required for the sample preparation. The intracellular concentration of 8-oxo-dGTP was determined in the range of 0.006 pmol/million - 0.010 pmol/million cells in normal or treated U2OS cells which correspond to 1.5 nM to 2.5 nM based on the U2OS cell volume of 4 L/million cells [21]. These concentrations were three orders of magnitude lower than physiological dGTP levels. These data prompt consideration of whether 8-oxo-dGTP plays any significant physiological roles at these low concentrations in eukaryotic cells. Quantification of the 8-oxo-guanine in DNA cannot conclude the contributions from the incorporation of 8-oxo-dGTP since oxidation of guanine base can occur in both nucleic acid strands and nucleotide pools. It was predicted from the frequency of mutations in the

presence of various concentrations of 8-oxo-dGTP *in vitro* that 2.4 nM 8-oxo-dGTP may increase the dA to dC mutation and indeed we found an increase in frequency by 6.6-fold in rodent liver mitochondria, however, the consequences of this increased mutation rate are unclear [20].

Under normal conditions, the intracellular levels of 8-oxo-dGTP and 8-oxo-GTP were not affected by shRNA-mediated knockdown of MTH1 in U2OS cells. Because insufficient number of the cells were used in the H_2O_2 experiment, the 8-oxo-dGTP levels were unmeasurable both with and without H_2O_2 treatment. However, the 8-oxo-GTP levels increased by approximately10-fold to 17-fold upon treatment with H_2O_2 confirming that ROS can markedly increase the intracellular oxidized nucleotide levels. Following H_2O_2 treatment, MTH1 knockdown resulted in little or no increase in 8-oxo-GTP (~25%). MTH1 knockdown did not affect the 8-oxo-GTP levels without H_2O_2 treatment. These results suggest that MTH1 may maintain low levels of 8-oxo-dGTP under normal conditions, and even after knockdown, it may take considerable time to form measurable levels of 8-oxo-dGTP. Consistently, the MTH1 mediated reaction is anticipated to be a slow process because the Michaelis constant, K_m for 8-oxo-dGTP with human MTH1 is reported to be approximately 10 uM to 20 uM, and the intracellular levels of the oxidized nucleotides are approximately 4 orders of magnitude lower than the K_m values [28,29]. In addition, the efficiency of 8-oxo-GTP cleavage by MTH1 is estimated to be approximately 2% of that of 8-oxo-dGTP [30]. Therefore, the physiological relevance of MTH1 in detoxification of 8-oxo-dGTP and/or 8-oxo-GTP in eukaryotic cells remains unanswered. These results indirectly support the hypothesis that alternate nucleotide detoxification mechanisms may exist in eukaryotic cells and further call into question the targeting of MTH1 for cancer [25].

Conclusion

Understanding the intracellular levels of oxidized nucleotides is essential to assess the frequency of mutations and DNA damage under physiological conditions. The LC-MS/MS-based analytical method to quantify the intracellular levels of 8-oxo-dGTP described in this work warrant further mechanistic studies to assess the potential of MTH1 inhibition as an anticancer target.

Conflict of Interest

All the authors are current or former employee of Gilead Sciences.

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