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Research Paper

A LC-MS/MS Method for the Analysis of Intracellular Nucleoside Triphosphate Levels

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Purpose. To simultaneously quantify intracellular nucleoside triphosphate (NTP) and deoxynucleoside triphosphate (dNTP) pools and to assess their changes produced by interfering with ribonucleotide reductase (RNR) expression in leukemia cells.
Methods. A HPLC-MS/MS system was used to quantify intracellular NTP and dNTP pools.
Results. The assay was linear between 50 nM, the lower limit of quantification (LLOQ), and 10 μM in cell lysate. The within-day coefficients of variation (CVs, $n=5$) were found to be 12.0–18.0% at the LLOQ and 3.0–9.0% between 500 and 5,000 nM for dNTPs and 8.0–15.0% and 2.0–6.0% for NTPs. The between-day CVs ($n=5$) were 9.0–13.0% and 3.0–11.0% for dNTPs and 9.0–13.0% and 3.0–6.0% for NTPs. The within-day accuracy values were 93.0–119.0% for both NTPs and dNTPs. ATP overlapped with dGTP and they were analyzed as a composite. This method was applied to measure basal intracellular dNTPs/NTPs in five leukemia cell lines exposed to the RNR antisense GTI-2040. Following drug treatment, dCTP and dATP levels were found to decrease significantly in MV4-11 and K562 cells. Additionally, perturbation of dNTP/NTP levels in bone marrow sample of a patient treated with GTI-2040 was detected.
Conclusions. This method provides a practical tool to measure intracellular dNTP/NTP levels in cells and clinical samples.

KEY WORDS: dNTP/NTP levels; GTI-2040; LC-MS/MS.

INTRODUCTION

Ribonucleotide reductase inhibitors, such as hydroxyurea and antisense GTI-2040, and nucleoside analogs, such as cytarabine, interfere with DNA and RNA synthesis in normal and malignant cells, thereby triggering the apoptosis cascade. The potential cytotoxic mechanism of action of these drugs include the inhibition of biosynthesis of deoxynucleoside triphosphates (dNTPs) and nucleoside triphosphates (NTPs), competition with endogenous dNTPs/NTPs for incorporation into DNA or RNA strands, or depletion of substrates critical for DNA/RNA syntheses. All these may result in perturbation of endogenous dNTP or NTP pools. Therefore, the determination of cellular dNTP and NTP levels is of fundamental importance in understanding the mechanisms of these agents, monitoring the treatment outcome, and development of chemo-resistance.

A number of analytical methods have been reported to quantify endogenous dNTPs (1–17) each with its advantages and disadvantages. Although enzymatic DNA polymerase assays are sensitive (1–4), they are hampered by interference with dideoxynucleoside triphosphates (ddNTPs). Radioimmunoassays (RIA) (5,6), although sensitive, cannot simultaneously measure multiple dNTPs and NTPs due to their cross-reactivity and interference with structurally similar molecules, such as 2′-deoxythymidine 5′-diphosphate (dTDP) (5). HPLC-UV has become an attractive method for the measurement of dNTPs and NTPs (7–14). However, interference with baseline noise (8,10) and low sensitivity require the use of a large sample size. Additionally, the time-consuming sample extraction (9,13,14) and a long run time (>2 h) (7) have limited their application to a small number of samples. Furthermore, it is necessary to remove NTPs from cell extracts prior to dNTPs analysis in order to yield accurate results, since the contents of endogenous NTPs in mammalian cells are several orders of magnitude higher than the corresponding dNTPs (11,12,14). Although HPLC-MS/MS methods have been used for the specific measurement of intracellular dNTPs (15,16) with high sensitivity, no direct, simultaneous determination of endogenous dNTPs and NTPs in cell extract matrices has been previously published (15–17).

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The present study reports the development of a HPLC-MS/MS method, with a simple dilution method of cell extracts, for the direct separation and simultaneous quantification of intracellular dNTPs and NTPs. The method has been validated and applied to the determination of endogenous dNTP and NTP levels in five human leukemia cell lines before and two after drug treatment, and in bone marrow samples from a patient with acute myeloid leukemia (AML) treated with an antisense GTI-2040 in combination with cytosine arabinoside on a Phase II clinical trial.

MATERIALS AND METHODS

Chemicals and Reagents

All dNTP standards, 2′-deoxyadenosine 5′-triphosphate (dATP), 2′-deoxythymidine 5′-triphosphate (dTTP), 2′-deoxyguanosine 5′-triphosphate (dGTP), 2′-deoxycytidine 5′-triphosphate (dCTP) and NTP standards, adenosine 5′-triphosphate (ATP), uridine 5′-triphosphate (UTP), guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate (CTP), 2-chloroadenosine-5′-triphosphate (ClATP), *N*,*N*-dimethylhexylamine (DMHA), formic acid (FA, 90%), sodium azide, sodium periodate, deoxyguanosine, methylamine, rhamnose, sodium acetate and acid phosphatase were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol and acetonitrile (ACN) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water for HPLC analysis was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). An ATP determination kit was obtained from Invitrogen (Rockville, MD). GTI-2040 (5′-GGC TAA ATC GCT CCA CCA AG-3′) was provided by the National Cancer Institute (Bethesda, MD).

Instrumentation

The HPLC-MS/MS system used consisted of a Shimadzu HPLC system (Shimadzu, Columbia, MD) and SPD-M10A PDA detector (Shimadzu, Columbia, MD) coupled to a Finnigan (ThermoFinnigan, San Jose, CA) LCQ ion trap mass spectrometer. The HPLC system used consisted of two LC-10AT *vp* pumps, a SIL-10AD autosampler (Shimadzu, Columbia, MD). Semi-automatic tuning was used to optimize all relevant parameters with an infusion of a mixture of dNTP/NTP solution. The MS^2 mass spectra of each dNTP and NTP were acquired with appropriate optimal collision energies. Instrument control and data processing were performed using XcaliburTM software (version 1.2, ThermoFinnigan).

HPLC Chromatographic and Mass Spectrometric Conditions

Previously reported HPLC conditions (16) were adapted and coupled to the LCQ ion trap mass spectrometer for the analysis of dATP, dTTP, dGTP, dCTP, ATP, UTP, GTP and CTP. Briefly, the analysis was performed on a Supelcogel ODP-50, 150×2.1 mm, 5 μm particle size column (Supelco, Sigma-Aldrich, St. Louis, MO) coupled to a 3.5 μm Waters Xterra MS C_{18} 10×2.1 mm guard column (Waters Corp., Milford, MA). The eluents used consisted of mobile phase A (MPA) containing 5 mM DMHA in ultra-pure water buffered to pH 7 by 90% FA and mobile phase B (MPB) consisting of 5 mM DMHA in ACN (50:50, *v*/*v*). Gradient program was used for the separation and identification of dNTPs and NTPs at a flow rate of 0.2 mL/min. The program was initiated with 0–10% MPB from 0 to 3 min, 10–45% MPB from 3–28 min, 45–0% MPB from 28 to 28.5 min, and 0% MPB from 28.5 to 40 min. The injection volume was 50 μL. The autosampler temperature was set at 4°C throughout the analysis.

The LCQ ion trap mass spectrometer with an ESI source was operated in the negative ion mode. The LC effluent was introduced into the ESI source without split. The electrospray voltage was set at 3.2 kV and the temperature of the heated capillary was set at 250°C. The LCQ ion trap mass spectrometer was operated with a background helium pressure of 1.75×10^{-3} Torr, a sheath gas flow of 96 (arbitrary unit), an auxiliary nitrogen gas flow of 45 (arbitrary unit) and a capillary voltage of −30 V. The ion transitions at *m*/*z* 490.1→392.1, 481.0→383.0, 506.1→408.1, 466.0→368.1, 483.0→385.0, 522.3→424.0, 482.1→384.1, 540.0→441.9 for dATP, dTTP, dGTP/ATP, dCTP, UTP, GTP, CTP, and ClATP, respectively, were used in multiple reaction monitor (MRM) mode. Collision energy values were optimized to 22–28% for these transitions. All performance was controlled by Finnigan Xcalibur (version 1.2) software in a Windows NT 4.0 system.

Cell lines and Cell Culture Conditions

Human leukemia cell lines K562, NB4, ML-1, MV4-11 and THP-1 were used. All cell lines were cultured in RPMI 1640 media supplemented with L-glutamine (Supplied by Tissue Culture Shared Resource, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio), 1% Penicillin-Streptomycin (Gibco, Rockville, MD) and 10% fetal bovine serum (FBS) (Invitrogen, Rockville, MD). The cell lines were maintained at 37°C in a humidified, 5% CO_2 environment. Trypan blue dye method and a hemocytometer were used to determine cell counts.

dNTPs/NTPs Extraction

Intracellular dNTPs and NTPs were extracted as previously described with a modification that involved sonication to enhance deproteinization (3,16,18). Briefly, cells were counted and monitored for viability using trypan blue exclusion test before extraction. Cell pellets were washed with phosphate buffered saline (PBS) (Supplied by Tissue Culture Shared Resource, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio) and deproteinized with an addition of 1 mL 60% methanol. The resulting solution was vortex-mixed for 20 s, incubated at −20°C for 30 min and sonicated for 15 min in an ice bath. Cell extracts were centrifuged at 1,000×*g* for 5 min at 4°C. Supernatants were separated and dried under a stream of nitrogen. The residues were reconstituted with 200 μL of mobile phase A and vortex-mixed for 20 s. Cell extracts were centrifuged at 1,000×*g* for 5 min at 4°C. A 50 μL aliquot of the resulting supernatants was then injected into the LC-MS/MS system for dNTP measurement and for NTP measurement a separate 50 μL aliquot of the 20× dilution supernatants was used.

Cell Matrices Preparation and the Recovery Study

A previously reported dephosphorylation method (19) was used in cell matrix preparation. Briefly, K562, MV4-11, HL-1, THP and NB4 cells (2×10^8) were harvested and washed two times with ice-cold PBS. dNTPs and NTPs were extracted as described above. The residue was reconstituted in 8 mL 4°C MPA. Dephosphorylation of intracellular dNTPs and NTPs was achieved by addition of 16 U of acid phosphatase (type XA, Sigma; St. Louis, MO, USA) and 50 μL of 1 M sodium acetate, pH 4.0, followed by a 1-h incubation at 37°C. The resulting solution was incubated in a 100°C water bath for 20 min. After the temperature returned to room temperature, the solution was centrifuged at $1,000\times g$ for 5 min. The supernatant was stored at −80°C for further use for calibration standard preparation. The recovery of this method was evaluated in triplicate at three concentrations (50, 500 and 5,000 nM) by comparing the ratios of response of dNTPs and NTPs spiked in the treated cell matrix to the same in MPA (20).

Calibration Standards and Method Validation

All the calibration standards preparation and method validation were performed in cellular matrices prepared as described above. The stock solutions of dNTPs and NTPs were prepared by mixing the commercially available NTP and dNTP standards with ultra-pure water to a final concentration of 1 mM and stored at −80°C. The calibration standards were prepared by spiking various amounts of dNTP and NTP and a constant amount of the internal standard ClATP in 0.20 mL cell matrices. The linearity was assessed in the concentration range of 50–10,000 nM of dNTP and NTP mixtures. Within-day accuracy and precision were determined at 50 nM (low quality control, LQC), 500 nM (medium quality control, MQC), and 5 μM (high quality control, HQC) in five replicates each. The between-day precision was determined across three QCs at five different days. The accuracy was assessed by comparing the nominal concentrations with the corresponding calculated values based on the calibration curve. The specificity of assay was evaluated by monitoring the MRM of each dNTPs and NTPs in blank cell matrices coupled to the HPLC retention times.

Stability Study of dNTPs and NTPs in K562 Cell Matrices

In order to ascertain the integrity of dNTPs and NTPs during sample preparation, stability study was performed using the LC-MS/MS method as described above. Briefly, triplicates of dNTPs and NTPs (500 nM) were incubated in K562 cell matrices at 4°C at different time periods (0, 3, 6, 9, 12, 15, and 24 h). A 50 μL aliquot of the resulting mixture was then injected into the LC-MS/MS system for dNTP and NTP measurement.

Determination of Intracellular dNTP and NTP Pools After GTI-2040 Treatment in Human MV4-11 and K562 Leukemia Cells

MV4-11 cells (8×10^6) and 5×10^6 K562 cells were treated with different concentrations of GTI-2040 at 0, 1.0, 5.0, 10.0 and 20.0 μM for 24 h. After counting Trypan Blue dyed treated cells with the hemocytometer, cell pellets were prepared and washed with ice-cold PBS. dNTPs and NTPs were extracted and determined as described above.

Determination of Intracellular dNTP and NTP Pools in a Bone Marrow Sample of a Leukemia Patient

Bone marrow samples were obtained with informed consent from a patient, who was treated with GTI-2040 infusion for 144 h in combination with high dose cytosine arabinoside, at the Arthur G. James Cancer Hospital, the Ohio State University. Bone marrow samples were collected and cell density was determined from GTI-2040 treated patients before treatment on Day 1 during treatment. The frozen bone marrow samples were thawed in a water bath at 4°C for 5 min and centrifuged for 5 min at $1,000\times g$. The resulting cell pellets were harvested and washed twice with 1 mL PBS. dNTPs and NTPs were extracted and determined as described above for leukemia cells.

Data Analysis and Statistics

dNTPs and NTPs levels were calculated based on the standard curves. Differences of intracellular dNTPs and NTPs between drug-treated samples and untreated control were compared using a Student's *t* test. Student's *t* test was performed using JMP Statistical Discovery software (version 4.0.4; SAS Institute, Cary, NC). The significance level was set at $p=0.05$.

RESULTS

HPLC-MS/MS Assay of dNTPs and NTPs

The total ion chromatogram of dNTPs and NTPs is shown in Fig. 1A. As shown, CTP was eluted first followed by dCTP, UTP, GTP, dTTP, dGTP/ATP, dATP and the internal standard ClATP. The retention times of CTP, dCTP, UTP, GTP, dTTP, dGTP/ATP, dATP and ClATP were 18.89, 19.00, 19.76, 20.08, 20.68, 21.02, 21.70 and 24.66 min, respectively. Although none of these NTPs and dNTPs were baseline-resolved, it was expected that from the molecular weight and fragmentation patterns they would be mass-resolved using MRM with LC-MS/MS, except for dGTP and ATP, which are isobaric in mass.

Using a direct infusion of a mixture of 1 μM each of dNTPs and NTPs at 10 μL/min for 1 min, the average electrospray ionization mass spectra of dATP, dTTP, dGTP, dCTP, ATP, UTP, GTP, CTP, and ClATP exhibited several abundant ions at *m*/*z* 490.1, 481.0, 506.1, 466.0, 506.10, 483.0, 522.3, 482.1, and 540.0, corresponding to their respective deprotonated molecule ($[M-H]^-$), under negative ionization conditions (Fig. 1B). The deprotonated molecules were thus selected as the precursor ions and their collision activated dissociation (CAD) spectra (Fig. 2) were obtained subsequently with a predominant daughter ion for each ion under the optimized collision energy on the LCQ instrument. As shown, the $M\text{-}H^-$ ions of dATP, dTTP, dGTP, dCTP, ATP, UTP, GTP, CTP, and ClATP exhibited fragment ions at *m*/*z* 392.1, 383.0, 408.1, 368.1, 385.0, 424.0, 384.1, and 441.9,

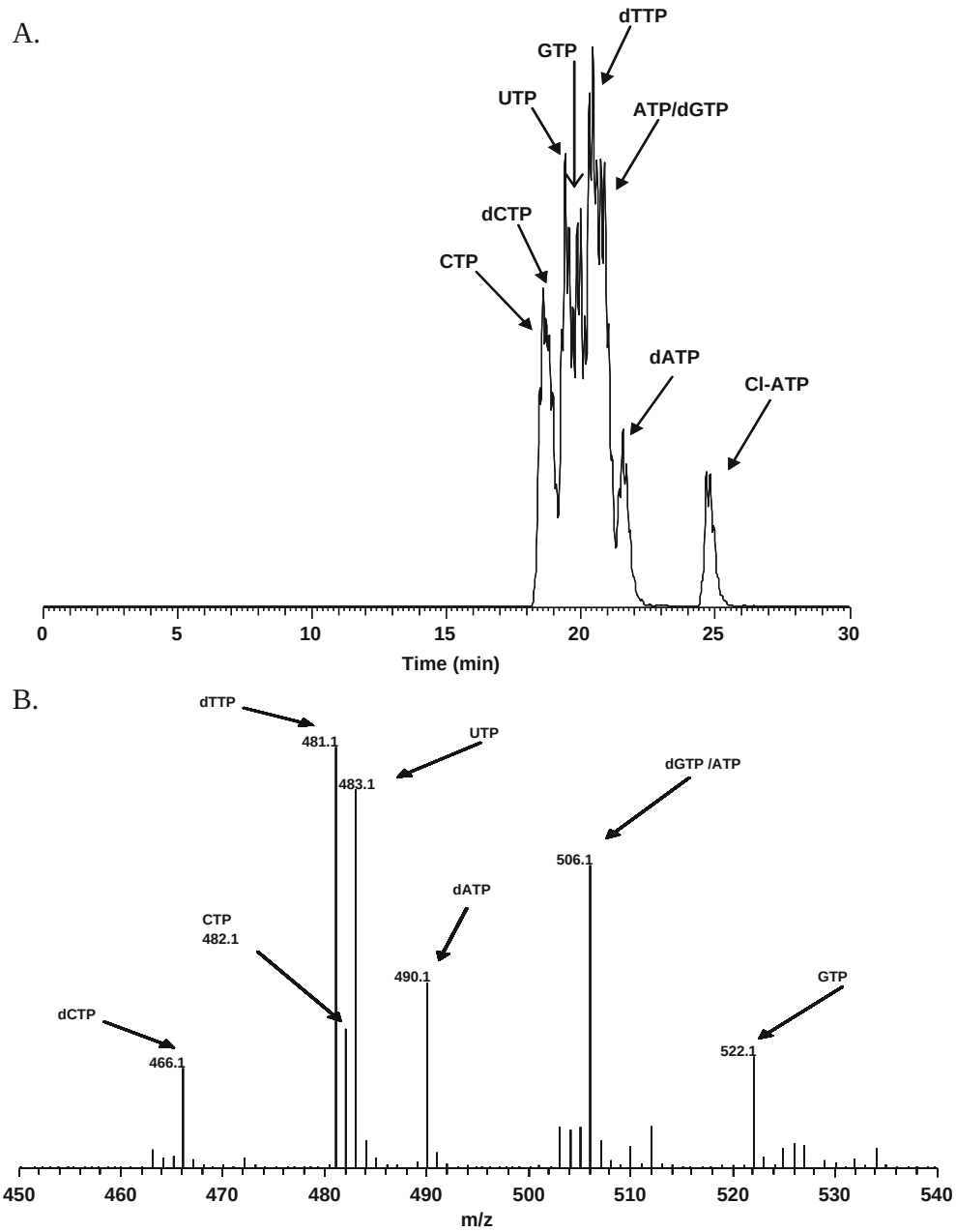

Fig. 1. **A** Total ion chromatogram of a standard mixture of ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, dTTP and ClATP. **B** Full scan of a standard mixture 1 μM of ATP, GTP, CTP, UTP, dATP, dGTP, dCTP and dTTP with a direct infusion at 10 μL/min.

respectively, corresponding to the removal of a phosphate group (98 Th) from their precursor ions. Therefore, the ion transitions at *m*/*z* 490.1→392.1, 481.0→383.0, 506.1→408.1, 466.0→368.1, 483.0→385.0, 522.3→424.0, 482.1→384.1, and 540.0→441.9 were selected for monitoring dATP, dTTP, dGTP/ATP, dCTP, UTP, GTP, CTP, and ClATP, respectively. These MRMs differ somewhat from the previously reported fragmentation patterns of dNTPs on an API 3000 (16), probably because of different type of instrument. Despite various attempts, dGTP/ATP were neither chromatographically nor mass-resolved and were analyzed as a composite.

Assay Validations and Recovery Studies

In order to accurately quantify intracellular dNTP and NTP levels, the assay was validated in K562 cellular matrices. For the construction of calibration curves, the cellular matrix was depleted of the endogenous dNTPs and NTPs by pretreating the K562 cell lysate with acid phosphatase to cleave the triphosphates. No extracted ion chromatogram peaks corresponding to dNTPs and NTPs were observed in the blank acid phosphatase-treated K562 cell extracts, suggesting that all dNTPs and NTPs in the cell extracts had completely been removed (Fig. 3B). Following spiking with a

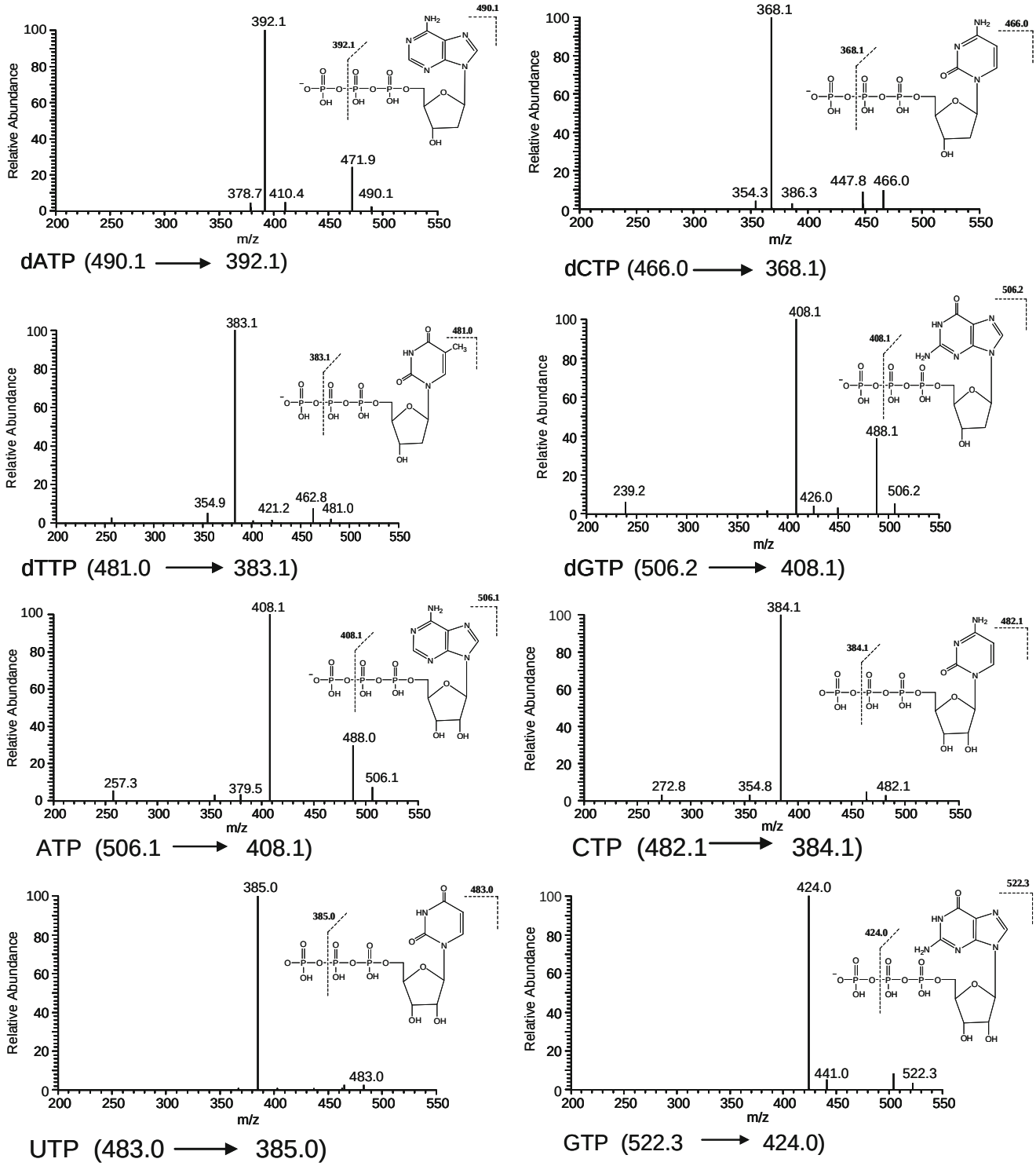

Fig. 2. Product ion mass spectra of the deprotonated molecular ions of dNTPs and NTPs.

dNTP and NTP mixture in the treated blank extract (Fig. 3A), several extracted ion chromatogram peaks were observed from 18 to 25 min, corresponding to the retention times of these nucleotides and deoxynucleotides. Good linearity was found for all dNTPs and NTPs at the range of concentration from 50 to 1,000 nM and from 1000 to 10000 nM with regression coefficients r^2>0.99 (Fig. 4). The intra- and inter-day precision values, expressed as %CV are summarized in Table I. The within-day coefficients of variation (CVs, n=5) were 15.5%, 15.1%, 11.7%, 18.0%, 14.4%, 7.92% and 14.9% for dATP, dTTP, dGTP/ATP, dCTP, UTP, GTP, and CTP, respectively, at the lower limit of

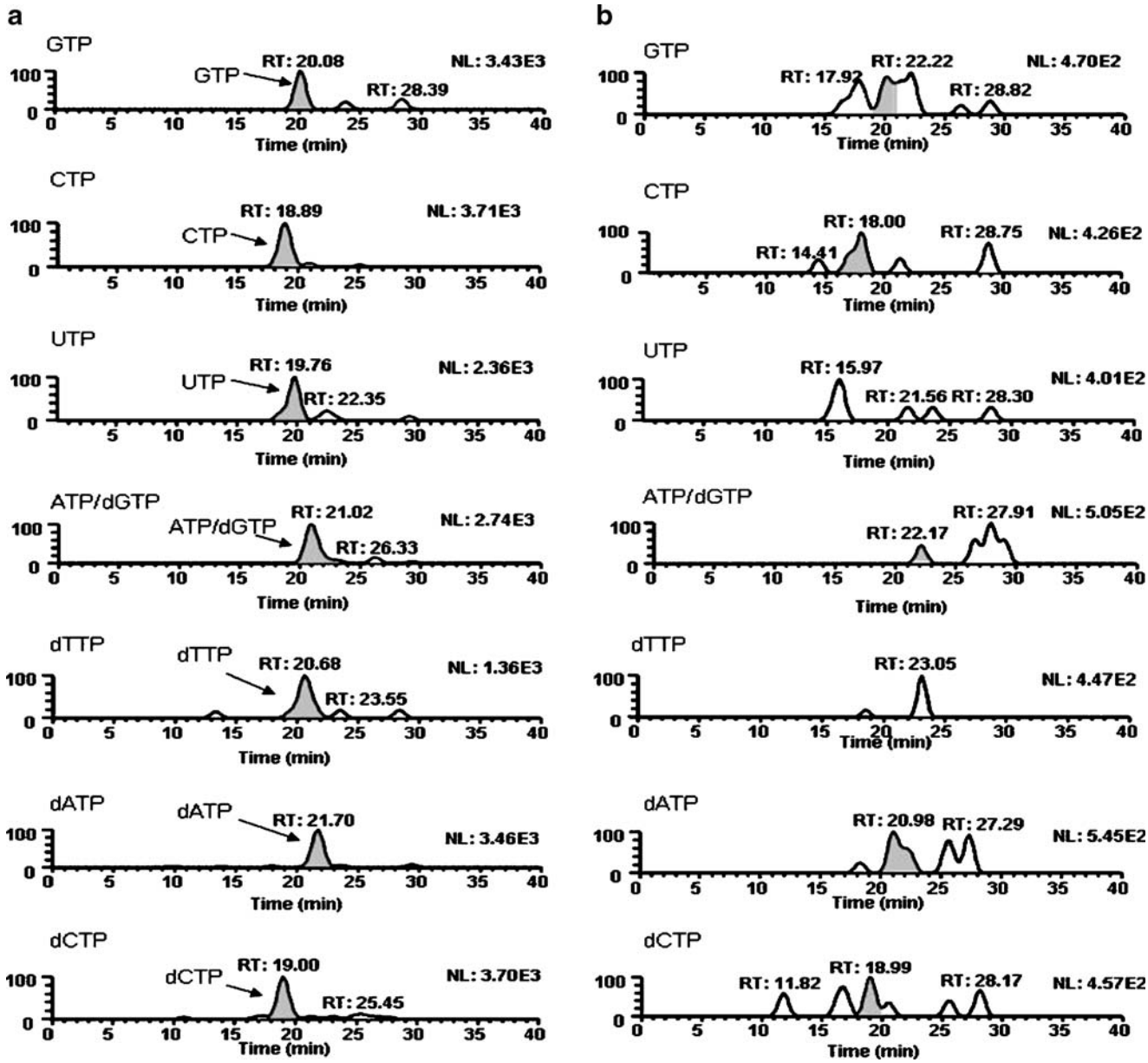

Fig. 3. **A** The extract ion chromatograms (*XIC*) of dNTPs and NTPs, 50 nM each, spiked into acid phosphatase-treated blank K562 cell extracts. **B** The XICs of dNTPs and NTPs in blank acid phosphatase-treated K562 cell extracts. No significant interference peaks were observed.

quantitation (LLOQ) of 50 nM and 3.0–9.0% between 500–5000 nM for dNTPs and 8.0–15.0% and 2.0–6.0% for NTPs. The between-day CVs (n=5) were 16.1%, 9.5%, 12.8%, 8.6%, 9.0%, 13.3% and 12.9% for dATP, dTTP, dGTP/ATP, dCTP, UTP, GTP, and CTP, respectively, at LLOQ and 3.0–11.0% between 500 and 5000 nM for dNTPs and 9.0–13.0% and 3.0–6.0% for NTPs. The within-day accuracy values were 93.0–119.0% for both NTPs and dNTPs. The recovery of this method among dNTPS and NTPs was listed in Table II, with a mean recovery value of about 64%.

Stability Study of dNTPs and NTPs in K562 Cell Matrices

The stability of dNTPs and NTPs in K562 cell matrices at a 24 h period is shown in Fig. 5. Student t test was performed to test the difference of mean values across different time period. No significant difference was found among mean values of dNTPs and NTPs at 95% confidence interval. Thus, dNTPs and NTPs are rather stable at 4°C for up to 24 h.

Analysis of dNTPs and NTPs in Different Cell Lines

The intracellular basal levels of dNTPs and NTPs in human leukemia cells K562, NB4, ML-1, MV4-11 and THP-1, 10×10^6 cells each, were measured using the above LC-MS/MS method. The concentrations of dNTP and NTP in these cells were calculated from standard curves constructed from individual acid phosphatase-treated cell extracts spiked with known dNTP and NTP concentration standards. The amounts of intracellular dNTP and NTP as expressed in pmol/10^6 cell are shown in Table III. The intracellular dNTP and NTP levels varied in different cell lines from 1–10, 1–5, 4–18, 550–1045, 85–

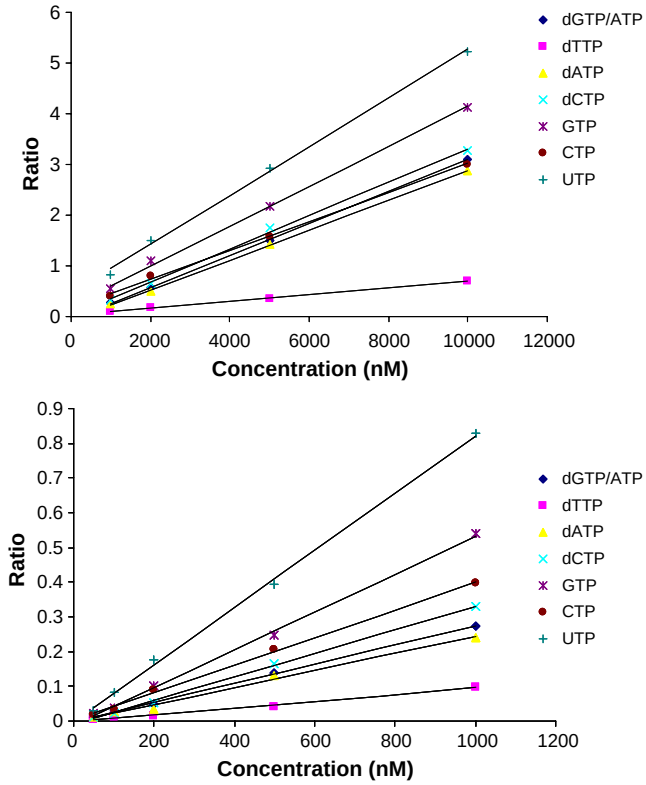

Fig. 4. Standard curves of dNTPs and NTPs in K562 cell matrices.

Table II. Recovery of dNTP and NTP Assay in Acidic Phosphatase-Treated K562 Cell Extracts Using the LC-MS/MS System

Analyte	Concentration (nM)	Recovery (%)[a]
dTTP	50	61.60±7.91
	500	68.65±3.27
	5,000	65.05±1.13
dATP	50	57.06±6.97
	500	68.65±4.97
	5,000	64.91±5.62
dCTP	50	65.95±5.03
	500	66.25±4.90
	5,000	68.45±2.37
GTP	50	57.39±8.15
	500	63.74±5.68
	5,000	68.92±3.86
CTP	50	63.36±4.35
	500	60.96±4.61
	5,000	71.67±2.25
UTP	50	54.93±9.18
	500	61.31±1.69
	5,000	67.97±0.56
dGTP/ATP	50	53.40±9.20
	500	66.77±1.62
	5,000	68.31±2.36

[a] Recovery was evaluated as mean area ratio of analyte in cell extract/analyte in mobile phase ± S.D (n=3)

330, 134–1,185 and 2,393–3,532 pmol/10^6 for dATP, dCTP, dTTP, GTP, CTP, UTP and dGTP/ATP, respectively. These values, not previously reported for these cell lines, are in the similar concentration ranges of 1.5–31, 0.7–27, 3–77, 316–800, 77–571, 87–1,726 and 915–2,627 pmol/10^6 for dATP, dCTP, dTTP, GTP, CTP, UTP and dGTP/ATP, respectively, as reported in previous studies for human lymphocytes, CEM-SS cell line, human A2780 ovarian, HT29 colon, K562 myelogenous leukemia, H322 non-small cell lung cancer cell lines and the murine lung cancer cell line Lewis Lung (2,10,21) (Table III).

Alterations in dNTPs and NTPs Levels in Leukemia MV411 and K562 Cells Treated with GTI-2040

Over-expression of ribonucleotide reductase, the highly regulated enzyme involved in the *de novo* synthesis of nucleoside triphosphates, has been found in almost every type of cancer. Down-regulation of ribonucleotide reductase activity has been considered an important strategy for anticancer therapy (22,23). GTI-2040 is a potent antisense inhibitor of the R2 subunit of the ribonucleotide reductase

Table I. Assay Validation Characteristics of dNTPs and NTPs in Cell Matrices by Negative ion ESI LC-MS/MS

		Reproducibility[a] (CV %, n=5)			Accuracy[a] (%, n=5)		
		Concentration (nM)					
	Analyte	50	500	5,000	50	500	5,000
Within-day	dTTP	15.1	4.17	3.87	107	101	101
	dATP	15.5	8.74	3.06	107	105	103
	dCTP	18.0	3.43	3.44	112	103	104
	GTP	7.92	1.97	3.88	111	92.8	102
	CTP	14.9	3.96	4.72	115	102	107
	UTP	14.4	5.52	6.08	105	95.7	109
	dGTP/ATP	11.7	3.15	3.60	119	98.7	103
Between-day	dTTP	9.5	8.2	7.4	–[b]	–	–
	dATP	16.1	7.7	4.6	–	–	–
	dCTP	8.6	10.7	2.6	–	–	–
	GTP	13.3	6.2	3.7	–	–	–
	CTP	12.9	5.1	4.1	–	–	–
	UTP	9.0	4.9	3.9	–	–	–
	dGTP/ATP	12.8	6.3	3.7	–	–	–

[a] Mean values; [b] Not determined

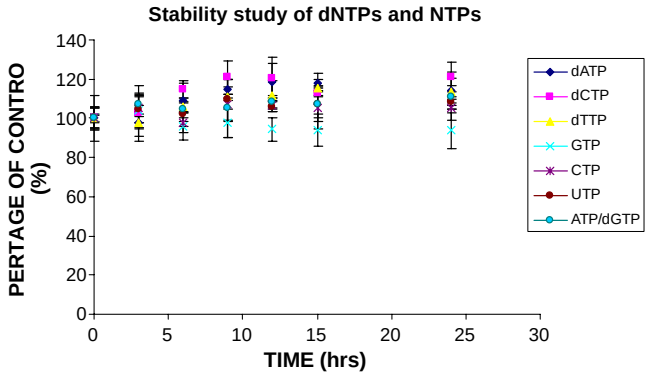

Fig. 5. Stability study of dNTPs and NTPs in K562 cell matrices.

(24,25). Therefore, monitoring of the perturbation of dNTP and NTP levels following GTI-2040 treatment is of critical importance for validation of its target, monitoring the treatment outcome, and the design of future combination therapies. Following GTI-2040 treatment in MV4-11 cells (Fig. 6), there were no significant changes among dGTP/ATP, GTP, CTP, UTP and dTTP levels at all exposure concentrations employed. However, dATP levels decreased by about 50% following exposure to 1 and 5 μM of GTI-2040 and became undetectable at 10 and 20 μM of drug treatment. dCTP level decreased drastically to an undetectable level following all four GTI-2040 treatment levels. In K562 cells, following GTI-2040 treatment, there were no significant changes in dGTP/ATP, GTP, CTP, and dTTP levels (Fig. 6). UTP levels increased by about 17–20%, following GTI-2040 exposures above 5 μM drug exposure and remained rather stable. dATP level decreased by about 50% at 20 μM of GTI-2040 exposure and dCTP level decreased by about 50% above 5 μM and remained the same. Thus, in this cell line, the modulation of dNTP and NTP levels did not appear to show a concentration dependent pattern, probably due to certain salvage or feedback regulations involved.

Determination of dNTPs and NTPs Levels in Bone Marrow Samples from a Leukemia Patient

Intracellular dNTP and NTP levels in bone marrow samples obtained from a leukemia patient as expressed in picomole per 10^6 cell are shown in Table IV. Following GTI-2040 infusion on day 1, dTTP and dCTP levels in bone marrow cells decreased from pre-treatment levels of 5.32 and 1.69 pmol/10^6 cells to 1.86 and 1.37 pmol/10^6 cells, respectively. The dATP level became undetectable from the pretreatment level of 7.42 pmol/10^6 cell. The pretreatment levels of dGTP/ATP, GTP, CTP and UTP were 851.71, 218.30, 105.74 and 296.04 pmol/10^6 cells, respectively, and these NTP levels increased slightly or moderately during the day 1 infusion.

DISCUSSION

The current method provides a simultaneous measurement of both dNTPs and NTPs in a single cell sample and only requires 5–10×10^6 cells. This method is rather simple, straightforward and employs an internal standard for the measurement, which compensates procedural loss. More importantly, this method employs dephosphorylated cellular matrix for the construction of the calibration curves rather than buffer or mobile phase as was used in most other previously published methods, providing a more realistic medium for the analysis.

Due to the presence of multiple phosphate groups, nucleotides are only weakly retained on reversed-phase HPLC columns under normal conditions. Therefore, ion-pairing agents are necessary to neutralize the negative charges of the nucleotides, which facilitate their retention on the reversed-phase columns. First, we tested a previously reported HPLC condition (16) using a gradient composing of 50% 6 mM DMHA dissolved in 20 mM ammonia formate and 50% ultrapure water as MPA and 50% solution A and 50% ACN as MPB. However, no separation of NTPs and dNTPs was achieved. We then lowered the DMHA concentration in the mobile phase to 5 mM, which resulted in a better resolution. Measurement of the pH of 5 mM DMHA aqueous solution showed a pH value of 10.3. When we evaluated their separation at the pH values of MPA from 5 to 10, the separation of eight dNTPs and NTPs was achieved at pH 7.0. Thus, we selected 5 mM DMHA as an ion-pairing agent. DMHA appears to increase the nucleotides binding to the column and prolongs their retention, resulting in better resolution of NTPs and dNTPs, higher signal intensity, lower interference, and is compatible with the use in ESI-MS (16,17,26,27). The pH value of MPA (5 mM DMHA, pH 10.3) was adjusted to 7.0, which is critical for nucleotide separation and quantification (17,27,28). At pH values above

Table III. Basal Levels of dNTPs and NTPs in Five Human Leukemia Cell Lines

	Concentration, pmol/10^6 cell (n=3, SD)						
Cell line/Analyte	dGTP/ATP	dATP	dCTP	dTTP	GTP	CTP	UTP
MV411	2642.9 (219.3)	1.53 (0.34)	1.93 (0.58)	7.56 (0.63)	607.8 (20.4)	165.0 (11.8)	315.6 (17.3)
K562	3,532.1 (123.6)	2.7 (0.96)	4.8 (0.77)	17.6 (7.23)	1045.0 (58.4)	329.6 (46.2)	1184.7 (67.6)
HL-1	2,837.4 (255.9)	10.1 (1.12)	1.44 (0.17)	7.14 (0.38)	550.6 (24.1)	116.8 (7.03)	290.6 (25.7)
THP	2,393.7 (180.3)	1.87 (0.37)	1.01 (0.41)	4.39 (0.17)	532.9 (39.2)	85.2 (16.9)	133.8 (7.8)
NB4	2,767.4 (271.0)	6.98 (0.62)	2.21 (0.39)	5.04 (0.98)	582.7 (67.8)	137.2 (19.3)	302.1 (26.4)
Literature values[a]	915–2,627	1.5–31	0.7–27	3–77	316–800	77–571	87–1726

[a] Range compiled from the following cell lines: human lymphocytes, CEM-SS, human A2780 ovarian, HT29 colon, H322 non-small cell lung cancer, murine Lewis lung based on (2,10,21)

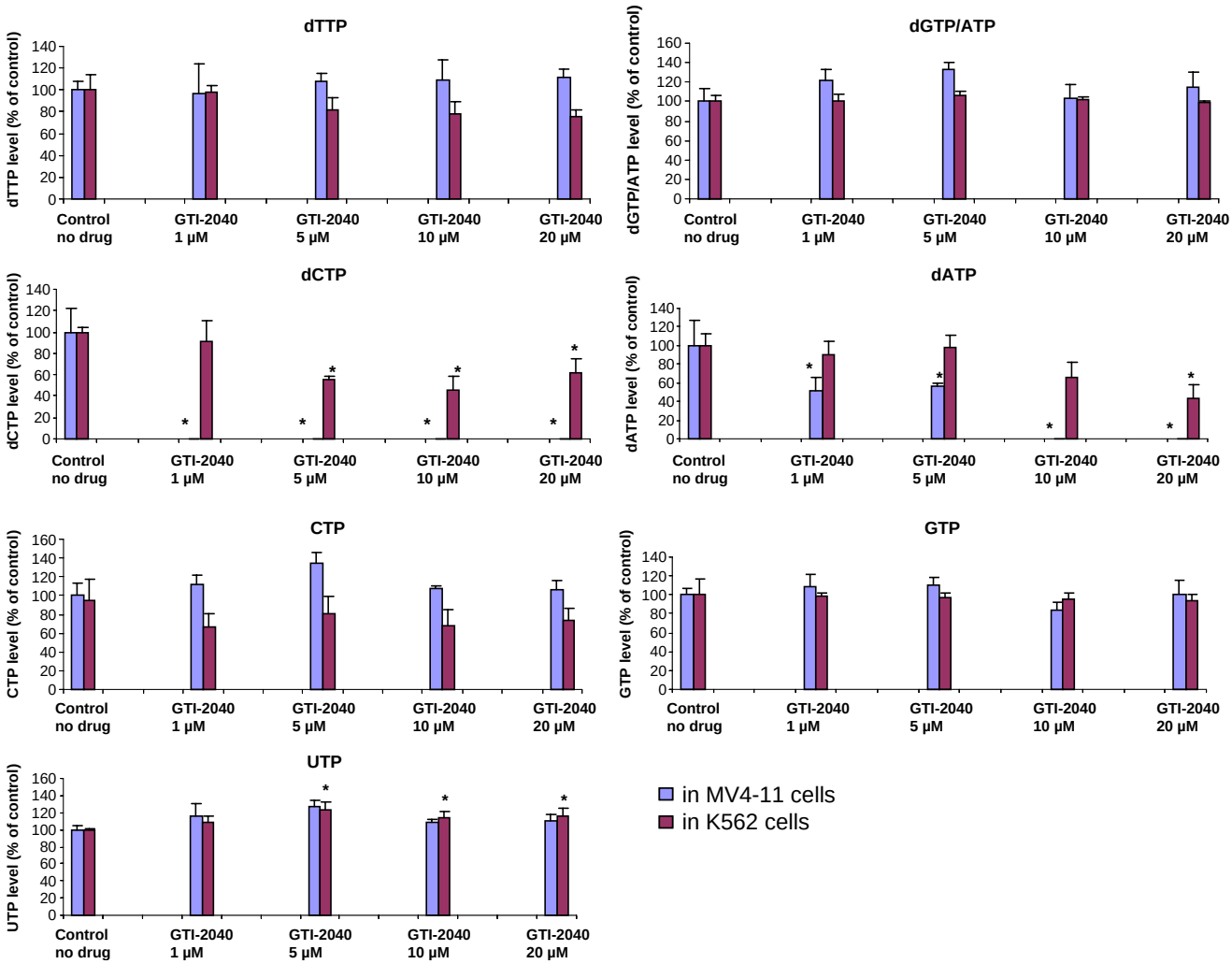

Fig. 6. **A** Alteration of dNTP and NTP levels in MV4-11 cells (*blue column*) or K562 cells (*red column*) following GTI-2040 treatment at various concentrations for 24 h (n=3). *Asterisks* indicate significant difference from untreated control at $p<0.05$.

7.5, nucleotide peak shape distortion was observed and signal intensity was significantly decreased, and at low pH, decreased signal intensity of some dNTPs and NTPs was also found.

dGTP and ATP are isobaric (identical mass) and give similar fragmentation patterns and retention times. This makes it impossible to differentiate these two compounds under the current LC-MS/MS condition, and the similar problem existed in a previous method (16). To overcome this limitation, attempts were made to eliminate the ATP levels. Two sample preparation methods were evaluated, boronate affinity chromatography (14,29) and degradation of the *cis-diol* NTPs by periodate and methylamine (30–32). However, the high salt concentration in the mobile phase for boronate affinity chromatography caused severe ion suppression and limits its application to LC-MS/MS. Additionally, we found that boronate affinity chromatography cannot completely deplete ATP. Since the average intracellular ATP level is about 2,000 pmol/10^6 cells (10,33) and the average intracellular dGTP level is only about 10 pmol/10^6 cells (7,10,33). Even 99% depletion (1% ATP remaining) would pose significant interference with the dGTP signal. A periodate oxidation method was evaluated and we found that ATP could not be completely depleted either. In addition, this procedure was reported to cause partial loss of

Table IV. dNTPs and NTPs Levels in Bone Marrow Samples from a Leukemia Patient

	Concentration, pmol/10^6 cell (SD)						
Sampling time/Analyte	dGTP/ATP	dATP	dCTP	dTTP	GTP	CTP	UTP
Pre-treatment	851.71	7.42	1.69	5.32	218.30	105.74	296.04
Day 1 during infusion	1,284.30	–[a]	1.37	1.86	406.71	164.60	401.59

[a] Undetectable

dGTP because of the formation of some dicarbonyl compounds (29). Although Hennere *et al.* (16) reported an improved periodate oxidation method by an addition of deoxyguanosine, which is supposed to circumvent the loss of dGTP; we were unable to reproduce their method. Additionally, the high salt concentration present in the final solution made it difficult to run a large number of samples on our LC-MS/MS system. Hence, we sought an enzymatic method using luciferin and luciferase; however, this method also caused partial dGTP loss and was not cost-effective due to the large amount of costly luciferin and luciferase required to eliminate ATP. Another possible method is to use a sodium azide oxidation method to deplete ATP was made (34). Unfortunately, this method also could not totally deplete ATP. Finally, we attempted to chromatographically resolve ATP from dGTP with an alternate solvent system. Although using ammonium acetate, we were able to resolve dGTP from ATP, the sensitivity of detection was found to be significantly reduced; therefore, we elected to use the current conditions. Although dGTP is importance in intracellular signaling pathways, the separate determination of dGTP was not central in the present investigation of perturbation of dNTP and NTP pools by antitumor agents, as most antileukemia nucleoside analogs cause perturbation of mainly intracellular dCTP, dATP and CTP levels, less significant on dGTP. Our method successfully quantified all other dNTP and NTP levels.

To improve the sensitivity and specificity, MRM was used to monitor each selected molecule. It is noteworthy that the molecular weights of parent ions and corresponding daughter ions for dTTP (*m*/*z* 383.0), CTP (*m*/*z* 384.1) and UTP (*m*/*z* 385.0) differ by only one Th. The presence of isotopic peaks could potentially cause interference. However, the intensities of isotopic signal of dTTP, CTP and UTP are weak and in all cases the overlapping of isotopic peaks with the analyte was found to be <5% (data not shown). In addition, the difference in retention times between the concerned analytes, e.g. 2 min between dTTP and CTP and 1 min between CTP and UTP, and the use of narrow mass isolation width (1.0) for all ions reduces the interference potential of the isotope peaks.

It is worth mentioning that the fragmentation patterns of dNTPs on the LCQ instrument differ from that reported on an API 3000. A single phosphate (98 Th) moiety was cleaved from precursor ions of dNTPs on the LCQ, while an entire nucleoside monophosphate group was cleaved from the parent ions of dNTPs leaving the diphosphate as the negative ion, when an API 3000 is used. The reason is probably due to the use of helium as the collision gas for the LCQ, while argon gas was used for the API 3000, leading to a difference in collision energy. Alternatively, this difference in fragmentation patterns may be due to the difference in ionization mechanism (e.g. ion trap for LOQ *vs*. triple quadrupole for API 3000). Direct comparison of collision energy is not feasible because of the difference in units, e.g. volts for API 3000 and %E for LCQ. Based on the fragmentation pattern and lower cost of the instrument, we prefer to use the LCQ ion-trap instrument for quantitation of the endogenous or drug triphosphates, recognizing that the triple quadrupole mass spectrometers are more commonly used for quantitation of small molecules.

Ribonucleotide reductase is an important enzyme which catalyzes the conversion of ribonucleotides (ADP, GDP, UDP, and CDP) to their corresponding deoxyribonucleotides (dADP, dGDP, dUDP, and dCDP). As DNA replication in cells is highly coordinated with the synthesis of its precursors, dNTPs, the balance between dNTP and NTP pools is important for cell functions. This pool size varies with cell types and in mammalian cells the concentrations of dNTPs and NTPs are ~20–500 μM and ~3–10 mM (approximately equivalent to 10–250 and 1,500–5,000 pmol/10^6 cells, assuming 2×10^6 cells/μL cell volume (35)), respectively (36). Our dNTP and NTP pools data for five human leukemia cell lines are within these ranges and comparable to the values for several cell lines as cited in Table III. Since GTI-2040 is a potent ribonucleotide reductase inhibitor, the inhibition of ribonucleotide reductase may result in decrease dATP and dCTP levels and increase ATP, GTP, UTP and CTP levels. After GTI-2040 treatment in MV411 and K562 cells, a significant decrease in dCTP and dATP has been observed and this confirms the inhibition effect GTI-2040 on ribonucleotide reductase. However, the dTTP level remains unchanged, possibly due to its biosynthesis pathway independent of ribonucleotide reductase. No significant changes were observed among NTP levels except a slight increase in UTP levels in K562 cells. This effect may be due to presence of the salvage pathway of intracellular NTP biosynthesis, which compensates the increase in NTPs. The significant decrease in dNTP and increase in NTP levels during GTI-2040 treatment in a leukemia patient based on our method also supports the potential mechanism of action of GTI-2040. However, more patient data is needed to confirm the pharmacological action of GTI-2040. Since GTI-2040 significantly decreases dCTP and dATP levels, the combination treatment with GTI-2040 and cytarabine may be a good strategy for future anticancer therapies, since the active metabolite of cytarabine, araCTP, competes with dCTP for DNA incorporation. This combination therapy is currently being evaluated at our institution with encouraging results (37).

CONCLUSION

In conclusion, we developed a non-radioactive LC-MS/MS method for quantifying intracellular NTP and dNTP pools simultaneously and successfully applied it to measure their levels in different cell lines and in bone marrow samples from a patient. Our present study provides a useful tool for the further measurement of biochemical modulations of nucleotide pools by various anti-cancer nucleoside analogs and nucleotide reductase inhibitors. Direct measurement of nucleotide pools provides important information for future combination therapies.

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