Analytical methodologies for quantitation of endogenous nucleotides (dGTP and dATP)

Materials and chemicals. Vacutainer® CPTs™ (cell preparation tubes; Becton Dickinson, Franklin Lakes, N.J.) were used for collection and separation of PBMCs from blood.

PBMC preparation of clinical samples. Blood samples (24 mL) for nucleoside analogue and corresponding endogenous nucleotides were collected in CPTs and processed in within 1 hour of collection. Each CPT was centrifuged at room temperature at 1,500 x *g* for 20 minutes and supernatant was transferred. PBMCs were washed using 0.9% NaCl solution at 4°C and cells were manually counted in duplicate using a hemacytometer. The PBMC mixture was then centrifuged at 400 x g for 10 minutes and supernatant removed. The cellular pellet was suspended with 1.5 mL of water/ methanol 30%/70% (vol/vol) at -20°C and lightly vortex and stored at -70°C until analysis.

Preparation of PBMC extracts. An aliquot of 50 μL of internal standards containing all of the following: 500 ng/mL 2-chloroadenosine triphosphate (2-Cl ATP), 250 ng/mL of ddC-TP and 250 ng/mL of ddGTP, was added to 500 μL cellular extract that was initially evaporated and then reconstituted with 1 mL of 0.1% formic acid immediately prior to solid phase extraction. Samples were fractionated using a weak anion exchange SPE cartridges (Waters Oasis WAX). The monophosphate and diphosphate anabolites were initially eluted with acidified 100 mM KCl while the triphosphorylated anabolite was eluted using alkalinized methanol. The collected samples tryphosphorylated samples were evaporated to dryness using a speed-vac. The residues were resuspended in 0.5 mL of 0.01% formic acid containing 1 unit of acid phosphatase (Sigma Biologicals, St. Louis, MO). Following 30 minutes incubation at 37°C, all of the phosphates were removed to yield the corresponding nucleoside analogs. The dephosphorylated samples were vacuum-dried and reconstituted with 50 μL of 5% methanol in deionized water and 30 μL of the samples were injected into a LC/MS/MS system for analysis.

Chromatographic and mass spectrometric conditions. Intracellular concentrations of deoxyadenosine triphosphate (dATP) and deoxyguanosine triphosphate (dGTP) were analyzed on a LC/MS/MS system composed of an Agilent 1100 HPLC system linked to a Applied Biosystems API 3000 mass spectrometer (Applied Biosystems, Foster City, CA). The analytes were separated using a reversed phase ACE C18 column (Advanced Chromatography Technologies) with the dimension of 2 x 50 mm and 3 μM packing. A step gradient program was applied to separate all the analytes. The mobile phase consisted methanol as component A and 20 mM ammonia acetate buffer at pH 4.5 as component B. The initial mobile phase was set at 7% methanol for three minutes, escalated to 15% methanol rapidly and maintained for 7 minutes. The mobile phase was changed to 80% methanol for two minutes and subsequently reduced to 7% methanol and equilibrated for another 11 minutes. After separation, the analytes in HPLC efferent was introduced into mass spectrometer through a turbo ion spray interface, in which analytes and internal standards were ionized and carried a positive charge. In addition, a heated turbo nitrogen stream was used to evaporate solvents and to increase ionization efficiency

Validation of the LC/MS/MS method. Six calibration standards were processed simultaneously (**Table 1**). Least-square linear regression using a weighting of $1/x^2$ was performed to establish a linear calibration curve between the area ratios of analyte to internal standard and the concentrations of analyte. The linearity was established by the back calculate concentration for each calibration standard. The comparison of the actual concentration to the expected theoretical value established the precision and accuracy of the assay. The linear dynamic ranges for dATP and dGTP were from 1.02 to 102 and 0.986 to 98.6 pmol/10^6 cells, respectively. The lower limit of quantification (LLQ) was determined to be 1.02 and 0.986 pmole/10^6 cells for dATP and dGTP, respectively.

Table 1.

Intracellular dATP concentrations (pmole/10^6 cells)					
Target value	1.02	5.09	10.2	50.9	102
N	6	6	5	6	5
Average	0.963	5.19	10.3	51.4	107
% CV	9.26	12.8	11.3	11.6	13.5
Intracellular dGTP concentrations (pmole/10^6 cells)					
Target value	0.986	4.93	9.86	49.3	98.6
N	6	5	6	6	5
Average	0.988	4.48	10.5	48.3	101.7
% CV	2.00	8.16	7.15	10.4	11.5

Selectivity and specificity

To determine whether mono- or di-phosphate analytes interfered with the assays, the selectivity of the weak anion exchange SPE step was demonstrated in validation study, in which dGTP at 5 ng alone and with the presence of 10 ng of dGMP and dGDP was used in the experiment. Samples were prepared in duplicates and undergone the same extraction process. The presence of monophosphate and diphosphate in the sample did not create positive bias and the method was specific for the determination of intracellular dGTP.

Intraday variation

Five replicates of quality control samples were assayed to assess the intraday variation and precision of the assay for dATP (mean: $27.7 + 2.88$ pmol/10^6 cells) and dGTP (mean: $9.72 + 0.87$ pmol/10^6 cells). Results demonstrated that dATP and dGTP had 10.4% and 9.0% variation, respectively.