IMPDH - Whole Cell Assay

IMPORTANT: Client-specified alterations can be accommodated.

Aim

This service has been specially tailored to validate IMPDH inhibition by a given compound in cultured cells. This whole cell assay consists in extracting, identifying and quantifying by HPLC the intracellular concentration of guanosine nucleotides (GMP, GDP and GTP) and IMP in compound-treated cells. This service was validated with mycophenolic acid, ribavirin and mizoribin, recognized inhibitors of IMPDH. When applied for the study of nucleoside analogues (NA), this assay can also reveal the formation of their mono-, di-, and triphosphate forms, indicating that nucleoside analogues enter the cells and are readily phosphorylated by cellular kinases.

1st Example: Mycophenolic acid (MPA)

As illustrated by Figure 1, a 48h-incubation of Huh 7 cells with mycophenolic acid (Sigma-Aldrich, 5µM), a known inhibitor of cellular IMPDH, results in a dramatic depletion of cellular GTP. As expected, the intracellular concentration of GMP is lowered, while IMP concentration is increased. Table 1 and Figure 2 present results of quantification of nucleotide mono- and tri-phosphates in treated and untreated cells.

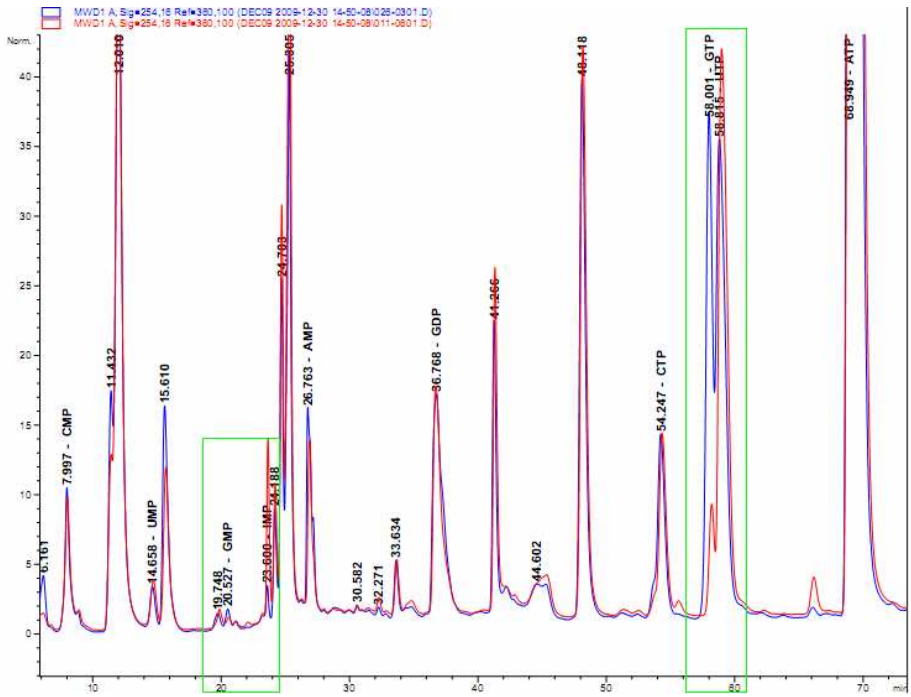
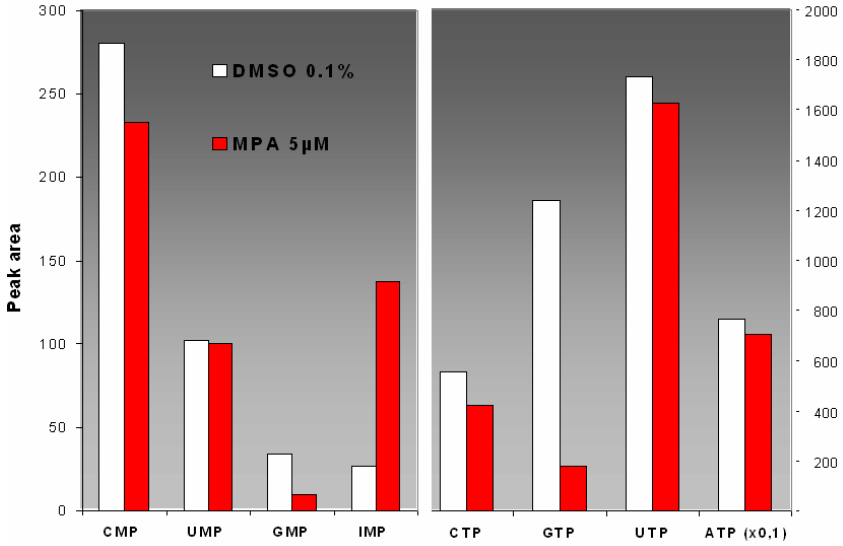


Figure 1. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 5µM MPA (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.

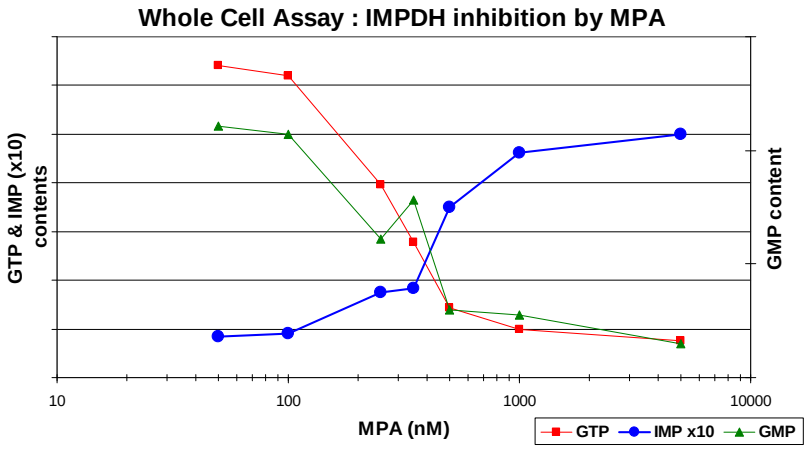


Concentration of nucleotides mono- and tri-phosphate in MPA- and DMSO-treated cells (measures as peak area, AU)

	DMSO 0.125%	MPA 5µM
CMP	280.2	233.2
UMP	102.2	100.2
GMP	33.8	9.6
IMP	27.0	137.2
AMP	396.6	273.2
CTP	554.0	424.8
GTP	1,237.0	182.0
UTP	1,734.8	1,627.0
ATP	7,665.0	7,057.0

Figure 2. Effects of 5µM MPA on cellular pool of nucleotide mono- and di-phosphates (results of quantification of HPLC spectra presented on Figure 1)

IC_{50} determination: Cellular GTP concentrations are plotted as a function of inhibitor concentration. IC_{50} is calculated using a standard four-parameter nonlinear regression analysis.
Plotting of minor nucleotides, such as IMP and GMP, is also available upon request.



2nd Example: Ribavirine (Rbv)

Numerous nucleoside analogues (NA) are currently used to treat viral infections. They are usually designed to inhibit one viral target. This remains in contrast with the observation that ribavirin, a purine nucleoside analogue currently used as a part of bi-therapy of hepatitis C infection, has multiple modes of action: (i) depletion of intracellular GTP pools by inhibition of the cellular IMPDH, (ii) inhibition of viral polymerase activity, (iii) induction of error catastrophe as a result of accumulation of mutations in the viral genome. Even if direct relationship between ribavirin antiviral action and IMPDH inhibition has not been demonstrated, the depletion of cellular GTP should result in increased frequency of ribavirin triphosphate incorporation by viral polymerase due to lower intracellular concentration of its natural competitor.

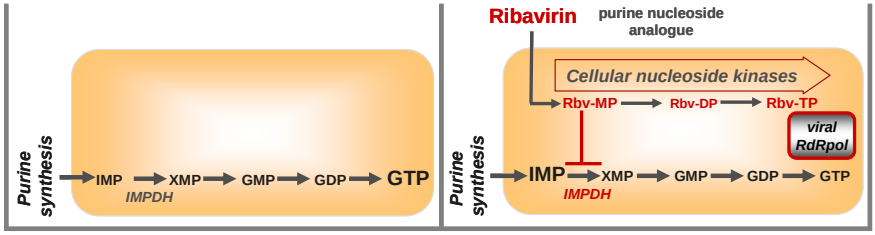


Figure 3. Modifications in cell-pool of nucleotides in Ribavirin-treated cells

To study the effect of nucleoside analogues on whole spectra of cellular purine and pyrimidine ribo- and deoxyribonucleotides, we have developed original cell-based analytical approach in which more than 31 (deoxy)ribonucleotides (mono-, di-, triphosphate) and nucleotide co-factors are extracted from cultured cells, separated by ion-pared chromatography and quantified. This cellular assay was validated with anti-viral and anti-cancer NA (ribavirin, gemcitabine) and known anti-metabolites (mycophenolic acid, leflunomide, hydroxyurea). In regards with new antiviral molecules identified in HCV cell culture systems (e.g. replicon), our cell-based assay allows to select the molecules of direct antiviral action from inhibitors of cell nucleotide biosynthesis.

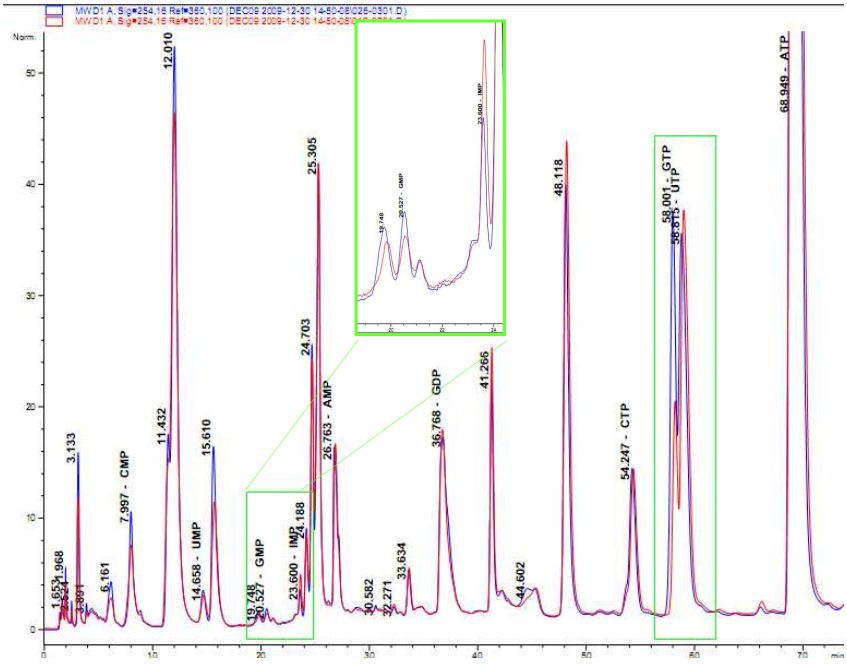
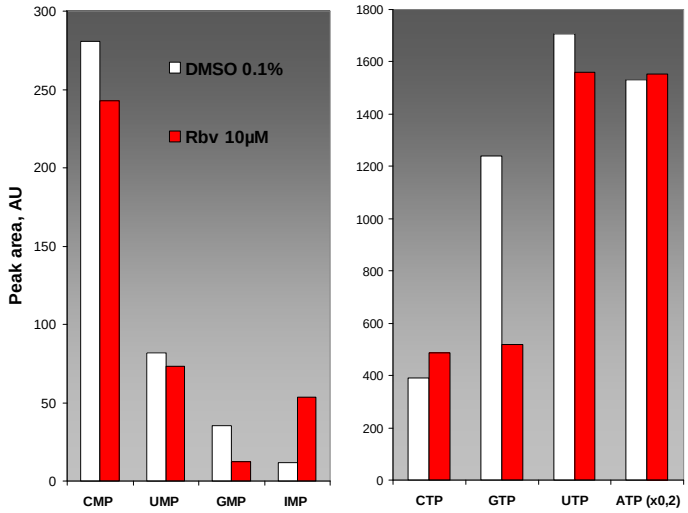


Figure 4. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 10µM Rbv (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.



Concentration of nucleotides mono- and tri-phosphate in Rbv- and DMSO-treated cells (measures as peak area, AU)

	DMSO 0.125%	Rbv 10µM
CMP	281.0	242.8
UMP	81.8	73.2
GMP	35.1	12.5
IMP	11.6	53.4
AMP	335.0	341.6
CTP	392.3	488.0
GTP	1,238.0	519.4
UTP	1,708.0	1,561.0
ATP	7,658.0	7,766.0

Figure 5. Effects of 10µM Rbv on cellular pool of nucleotide mono- and di-phosphates (results of quantification of HPLC spectra presented on Figure 4)

Materials & Methods

Cells treatment

Huh-7 cells are grown in an atmosphere of humidified 5% CO_2 at 37°C in DMEM medium supplemented with 2mM L-glu tamine, 10% heat-inactivated fetal bovine serum and streptromycin-penicillin. Exponentially grown Huh-7 cells are seeded at ~$6x10^5$ cells per 10cm cell-culture dish. After 48h of growth, the culture medium is replaced with fresh FCS-supplemented medium followed by addition of 10µL of DMSO or DMSO-dissolved compound.

Extraction of nucleotides and deoxynucleotides - Sample preparation

The nucleotides are extracted from cell monolayers by addition of 3 ml per dish of ice-clod 80% acetonitril for 1h. The extracts are centrifuged to remove cellular debris and nucleotides are extracted by SPE procedure (SAX column, Supelco, Sigma-Aldrich) pre-conditioned with methanol, water and acetonitrile. The eluent is filtered through 0.45µm filter membrane (Roth) and analyzed by HPLC.

Analytical system

1) An Agilent 1100 series liquid chromatograph fitted with binary pump G1312A, vacuum degasser G1322A, well-plate autosampler G1367A, thermostatted column compartment G1316A and multiple wavelenght and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.

2) Zorbax Extend-C18 4.6x150mm, 3.5µm particle size and corresponding guard column (Agilent).

5µl of cell extract were analyzed using Zorbax Extend-C18 column by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides[1] with slight modifications as follows.

HPLC calibration, peak identification and quantification

Calibrations are performed with standards prepared in mobile phase and with standards mixed with cell extracts, which are run immediately before and after every series of samples. Assignment of the peaks that correspond to different deoxyribonucleoside and ribonucleoside mono-, di-, and triphosphate of the cell extract spectrum is done by comparing both retention times and characteristics of UV absorption spectra (254/280 ratio) with those of standards. The area of individual peaks was measured using ChemStation software (Agilent).

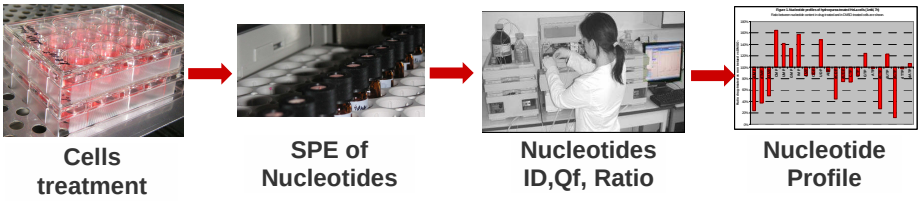
[1] D. Di Pierro, B. Tavazzi, C. Federico Perno, M. Bartolini, E. Balestra, R. Calio`, B. Giardina, G. Lazzarino (1995) **An Ion-Pairing High-Performance Liquid Chromatographic Method for the Direct Simultaneous Determination of Nucleotides, Deoxynucleotides, Nicotinic Coenzymes, Oxypurines, Nucleosides, and Bases in Perchloric Acid Cell Extracts** *Analytical Biochemistry* 231, 407–412

RNR inhibition - Whole Cell Assay

IMPORTANT: Client-specified alterations can be accommodated.

To study the effect of nucleoside analogues on the whole spectra of cellular purine and pyrimidine ribo- and deoxyribonucleotides, we have developed an original cell-based analytical approach in which more than 31 (deoxy)ribonucleotides (mono-, di-, triphosphate) and nucleotide co-factors are extracted from cultured cells, separated by ion-pared chromatography and quantified. These cellular assays were validated with anti-viral and anti-cancer NA (ribavirin, gemcitabine) and known anti-metabolites (mycophenolic acid, leflunomide, hydroxyurea, methotrexate).

Aim
"RNR inhibition: Whole Cell Assay" has been specially tailored to validate RNR inhibition by a given compound in cultured cells. This whole cell assay consists in extracting, identifying and quantifying by HPLC the intracellular concentration of deoxynucleotides di- and triphosphate in compound-treated cells. This service was validated with hydroxyurea and gemcitabine in HeLa cultured cells.



1st Example: Hydroxyurea (HU)

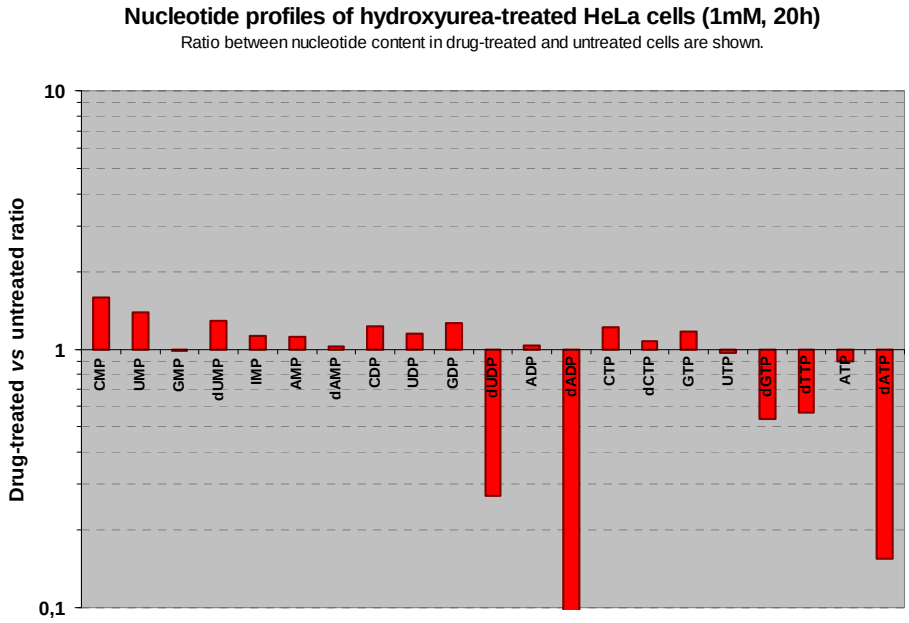


Figure 1: Nucleotide profiles of hydroxyurea-treated HeLa cells

Hydroxyurea is an antineoplastic agent, antimetabolite, used to treat melanoma, chronic myelocytic leukemia and certain blood disorders. Hydroxyurea is known to inhibit DNA synthesis by destroying the catalytically essential free radical of class I ribonucleoside diphosphate (rNDP) reductase, thereby blocking the *de novo* synthesis of deoxyribonucleotides. In mammalian cells, hydroxyurea treatment causes a differential depletion of the four deoxyribonucleoside triphosphate pools with dATP being most severely depleted [1, 2]. As illustrated by Figure 1, hydroxyurea treatment induces in HeLa cells profound depletion of deoxyadenosine triphosphate and significant loss of dADP, dUDP and dTTP, which is consistent with previously published data [1, 2].

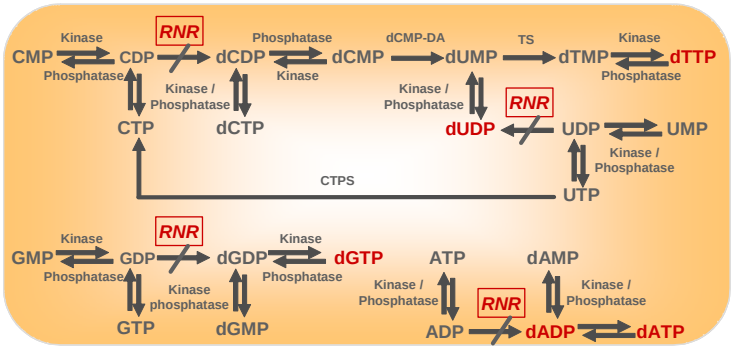


Figure 2. Effect of hydroxyurea on cellular pool of deoxynucleotides. The depleted nucleotides are shown in red. Ribonucleotide reductase (RNR), a recognized target of hydroxyurea, is framed in red.

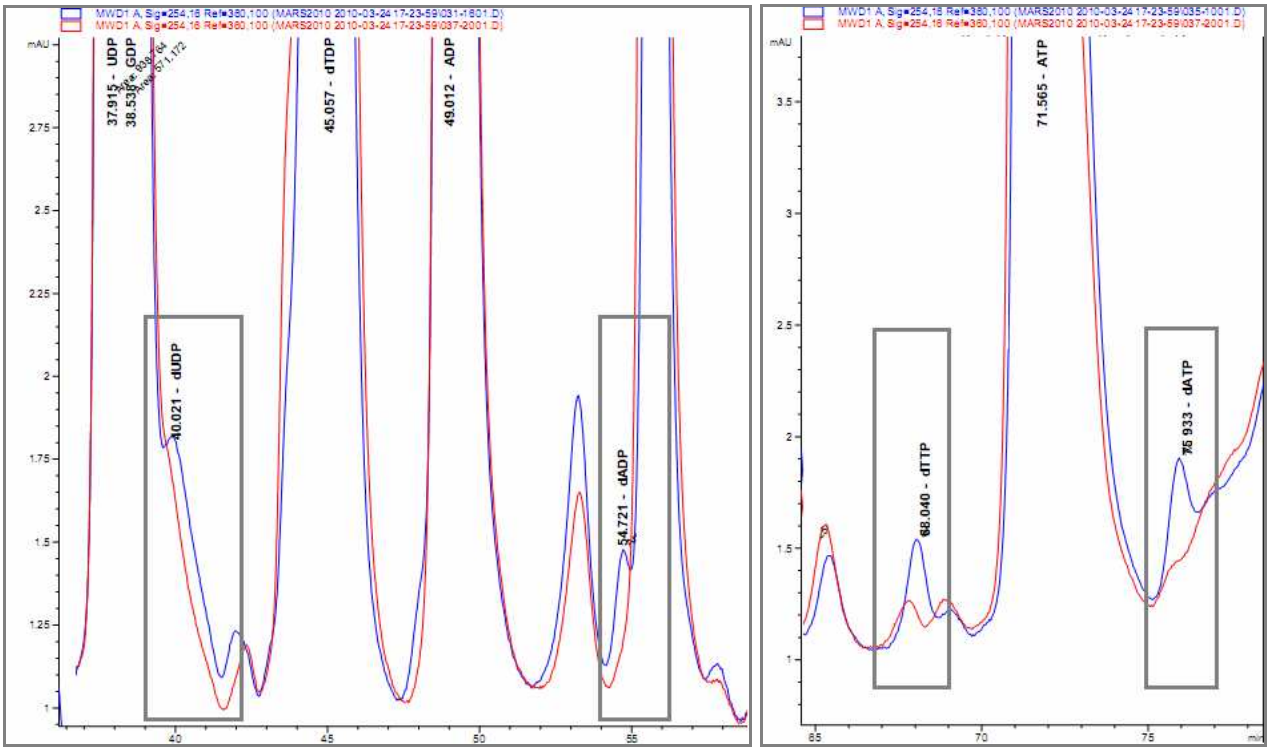


Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 1mM hydroxyurea (red) and DMSO (blue). Focus on depletion in dUDP and dADP is shown on left and in dTTP and dATP on right.

[1] Bianchi, V., Pontis, E., and Reichard, P. (1986) **Changes of Deoxyribonucleoside Triphosphate Pools Induced by Hydroxyurea and Their Relation to DNA Synthesis** *J. Biol. Chem*. 261, 16037–16042

[2] S. P. Hendricks and C. K. Mathews (1998) **Differential Effects of Hydroxyurea upon Deoxyribonucleoside Triphosphate Pools, Analyzed with Vaccinia Virus Ribonucleotide Reductase.** *J. Biol. Chem.* 273, 29519-523

2nd Example: Gemcitabine (dFdC))

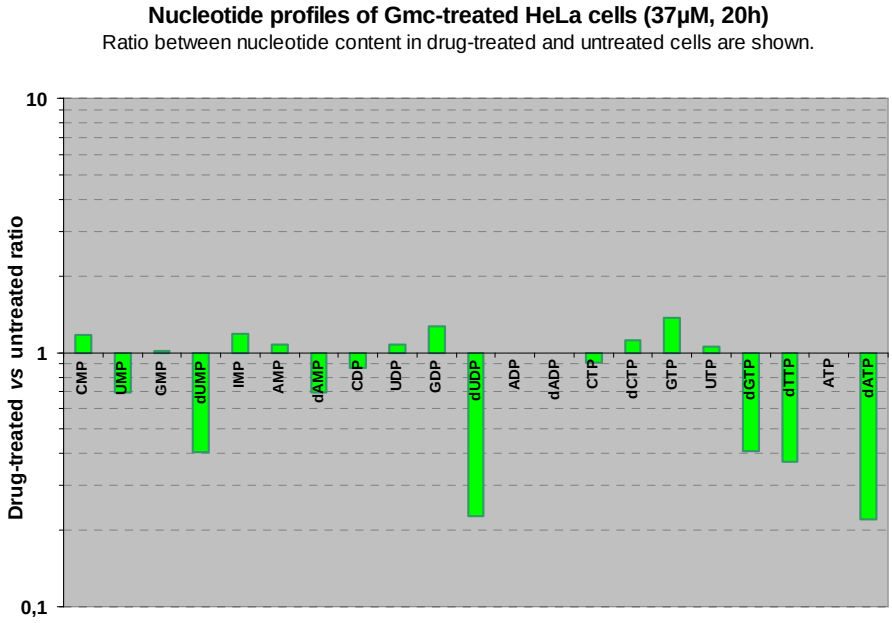


Figure 4: Nucleotide profiles of gemcitabine-treated HeLa cells

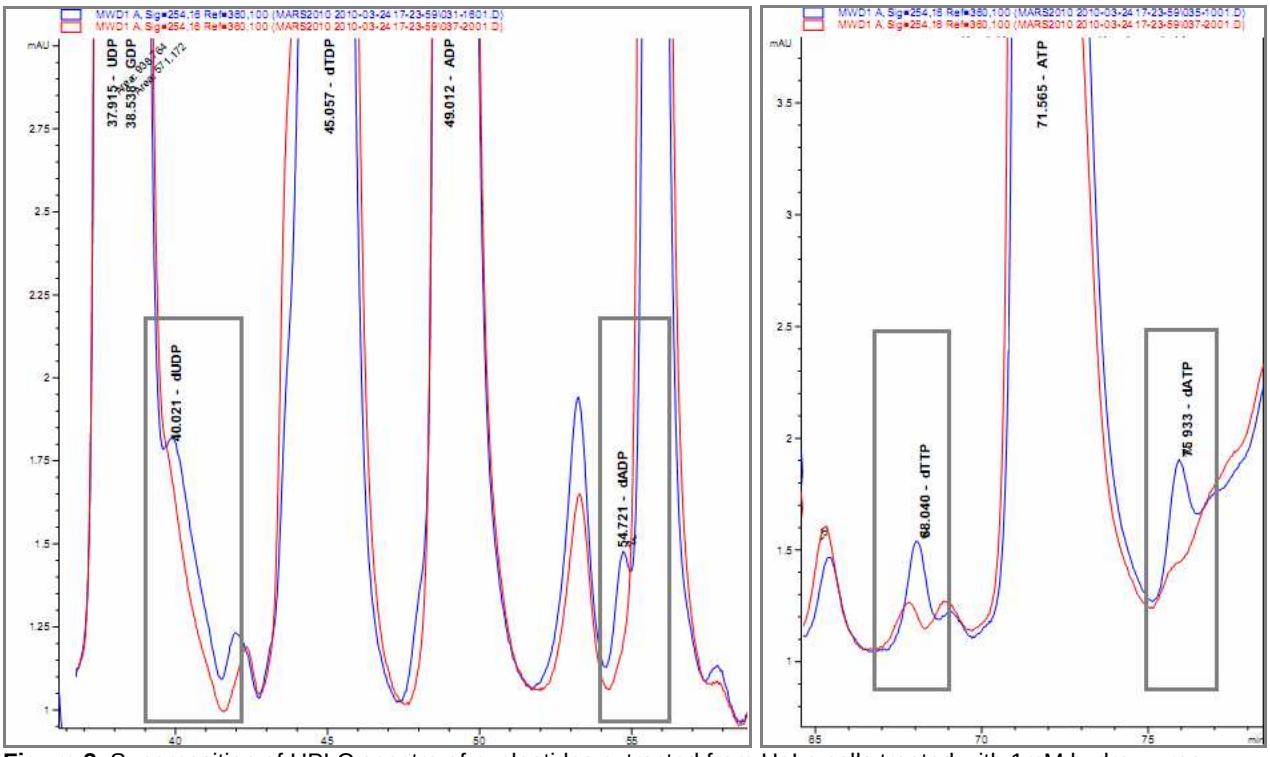


Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 1mM hydroxyurea (red) and DMSO (blue). Focus on depletion in dUDP and dADP is shown on left and in dTTP and dATP on right.

Gemcitabine (2',2'-difluorodeoxycytidine, **dFdC**) is a nucleoside analogue clinically used as an anticancer prodrug. Its phosphorylated metabolites target numerous cellular enzymes involved in nucleotide biosynthesis, including ribonucleotide reductase (RNR) which is strongly inhibited by diphosphorylated form of gemcitabine, dFdCDP. As shown in Figure 4, major changes in nucleotides induced by dFdC in HeLa cells concern the depletion in cellular dATP, dTTP, dGTP and dUDP due to RNR inhibition. The depletion of cellular dUMP indicates inhibition of dCMP-deaminase consistently with previously reported data[1], but may also reflect the decrease in cellular dUDP, a source of dUMP.

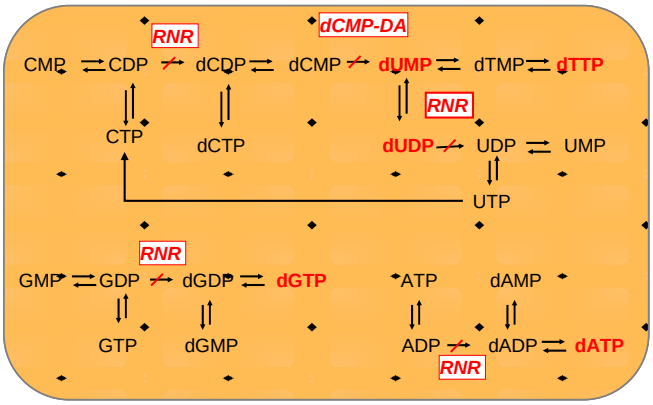


Figure 5. Effect of gemcitabine on cellular pool of nucleotides and deoxynucleotides. The depleted (<50% of control) nucleotides are shown in red. Ribonucleotide reductase (RNR) and dCMP-deaminase (dCMP-DA), recognized targets of gemcitabine, are framed in red.

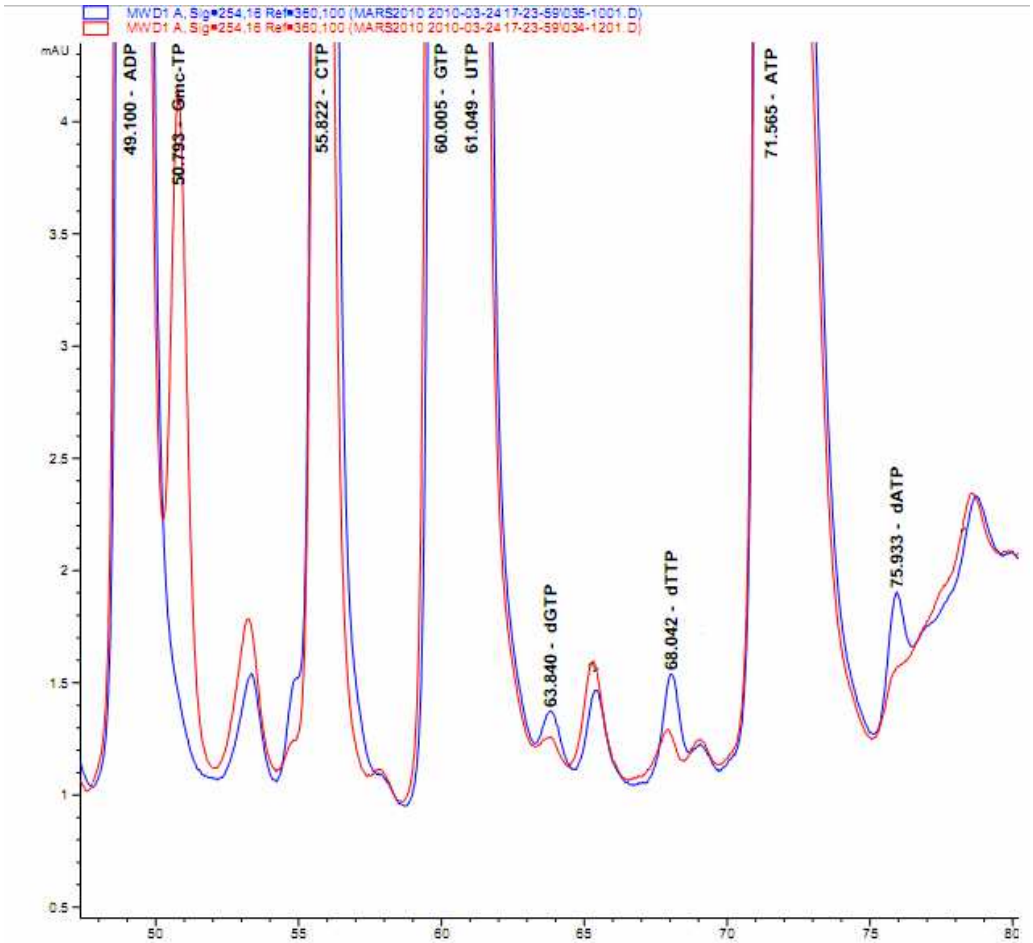


Figure 6. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 37µM gemcitabine (blue) and DMSO (red) illustrating depletion in dNTP.

[1] C. J.A. van Moorsel, A. M. Bergman, G. Veerman, D. A. Voorn, V. W.T. Ruiz van Haperen, J. R. Kroep, H. M. Pinedo, G. J. Peters (2000) **Differential effects of gemcitabine on ribonucleotide pools of twenty-one solid tumour and leukaemia cell lines** *Biochimica et Biophysica Acta* 1474, 5-12

Materials & Methods

Cells treatment:

HeLa cells were grown in an atmosphere of humidified 5% CO_2 at 37℃ in DMEM (PAA) medium supplemented with 2mM L-glutamine (Gibco/BRL), non essential amino acids (PAA), 10% heat-inactivated fetal bovine serum (BioWest) and streptomycin-penicillin (Sigma). Exponentially grown HeLa cells were seeded at ~$6x10^5$ cells per dish. After 48h of growth, the culture medium was replaced with fresh FCS-supplemented medium (10ml per Petri dish) followed by addition of 10µL of DMSO or DMSO-dissolved compounds. Six Petri dishes of cells per experiment were used to provide the nucleotide amount sufficient for UV-quantification of deoxynucleotides. At the end of a 7h-incubation, the medium was aspirated, cells monolayers washed twice with 5ml PBS, and used for nucleotides extraction.

Extraction of nucleotides and deoxynucleotides - Sample preparation:

The nucleotides were extracted from cell monolayers by the addition of ice-cold 80% acetonitril for 1h. The extracts were centrifuged to remove cellular debris and load on SAX column (100mg, Supelco) pre-conditioned with methanol, water and acetonitrile. Once sample was effused completely, the cartridge was washed with 3ml 80% ACN and 3ml water and eluted with 1M KCl. The eluent was filtered through a 0.45µm filter membrane (Roth) and analyzed by HPLC.

Analytical system:

1) Agilent 1100 series liquid chromatograph fitted with binary pump G1312A, vacuum degasser G1322A, well-plate autosampler G1367A, thermostated column compartment G1316A and multiple wavelength and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.
2) Zorbax Extend-C18 4.6x150mm, 3.5µm particle size and corresponding guard column (Agilent).
5µl of cell extract were analyzed using Zorbax Extend-C18 column by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides[1] with slight modifications as follows.
Peak identification of the different nucleoside mono-, di-, and triphosphates, was made from their characteristic UV absorption spectra and retention times compared with those of a mixture of standards (Sigma) run immediately before cell extracts. The area of individual peaks was measured using ChemStation software (Agilent).

HPLC conditions:

Nucleotides were analyzed by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides with slight modifications in pH and concentration of buffers adjusted to ensure adequate resolution of all nucleosides/nucleotides as follows: Buffer A: 20mM KH_2PO_4, 10mM tetrabutylammonium hydroxide pH 8.50; Buffer B: 100mM KH_2PO_4, 3mM tetrabutylammonium hydroxide, pH 3.0, 30% methanol. Flow rate: 1ml/min. Temperature constantly kept at 21℃. Gradient was formed as follows : 15 min at 100% buffer A; 5min at up to 90% buffer A, 5 min up to 70% buffer A; 15min up to 63% buffer A, 15 min up to 55% buffer A, 20min up to 45% buffer A, 10min up to 25% buffer A, 10min up to 0% buffer A. The spectra were recorded at 254 and 280nm.

HPLC calibration:

The calibration was performed with following standards: dUMP, dUDP, dUTP, dCDP, dCMP, dCDP, dCTP, dTMP, dTTP. dGDP and dGTP were not separated from unknown major peak and were not quantified. The standards prepared in Buffer A or those mixed with cell extracts were run immediately before and after series of samples. The data were used for calculation of retention times (Rf) and absorbance at 254nm and 280nm (254/280 ratio) specific for each nucleotide.

Peak identification and quantification:

5µl of cell extract were injected and nucleotides were separated as described before. Assignment of peak of the different deoxyribonucleosides and ribonucleosides mono-, di-, and triphosphate was done by comparing both retention times and characteristic UV absorption spectra (254/280 ratio) with those of standards. The area of individual peaks was measured using ChemStation software (Agilent).

Quality control:

The experiments are done in duplicates and relative standard deviation (RSD) is usually less than 12%.

[1] D. Di Pierro, B. Tavazzi, C. Federico Perno, M. Bartolini, E. Balestra, R. Calio`, B. Giardina, G. Lazzarino (1995) **An Ion-Pairing High-Performance Liquid Chromatographic Method for the Direct Simultaneous Determination of Nucleotides, Deoxynucleotides, Nicotinic Coenzymes, Oxypurines, Nucleosides, and Bases in Perchloric Acid Cell Extracts** *Analytical Biochemistry* 231, 407–412

Multi-targeted Antifolates - Whole Cell Assay

Inhibition of *de novo* purine and pyrimidine biosynthesis

IMPORTANT: Client-specified alterations can be accommodated.

To study the effect of nucleoside analogues on the whole spectra of cellular purine and pyrimidine ribo- and deoxyribonucleotides, we have developed an original cell-based analytical approach in which more than 31 (deoxy)ribonucleotides (mono-, di-, triphosphate) and nucleotide co-factors are extracted from cultured cells, separated by ion-pared chromatography and quantified. These cellular assays were validated with anti-viral and anti-cancer NA (ribavirin, gemcitabine) and known anti-metabolites (mycophenolic acid, leflunomide, hydroxyurea, methotrexate).

Aim

"Multi-targeted Antifolates: Whole Cell Assay" has been specially tailored to validate inhibition of *de novo* biosynthesis of purine and pyrimidne nucleotides by a given compound in cultured cells. After incubation of cultured cells with the inhibitor, nucleotides are extracted, separated, identified and quantified by UV-HPLC. This service was validated with methotrexate in HeLa cultured cells.

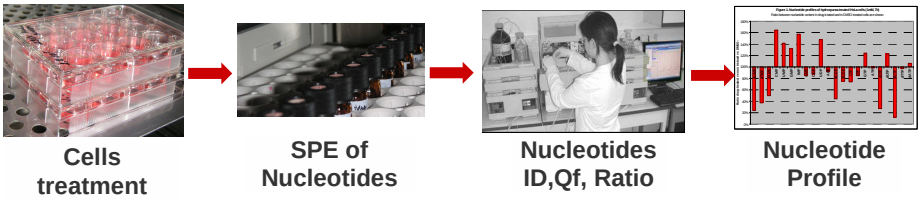
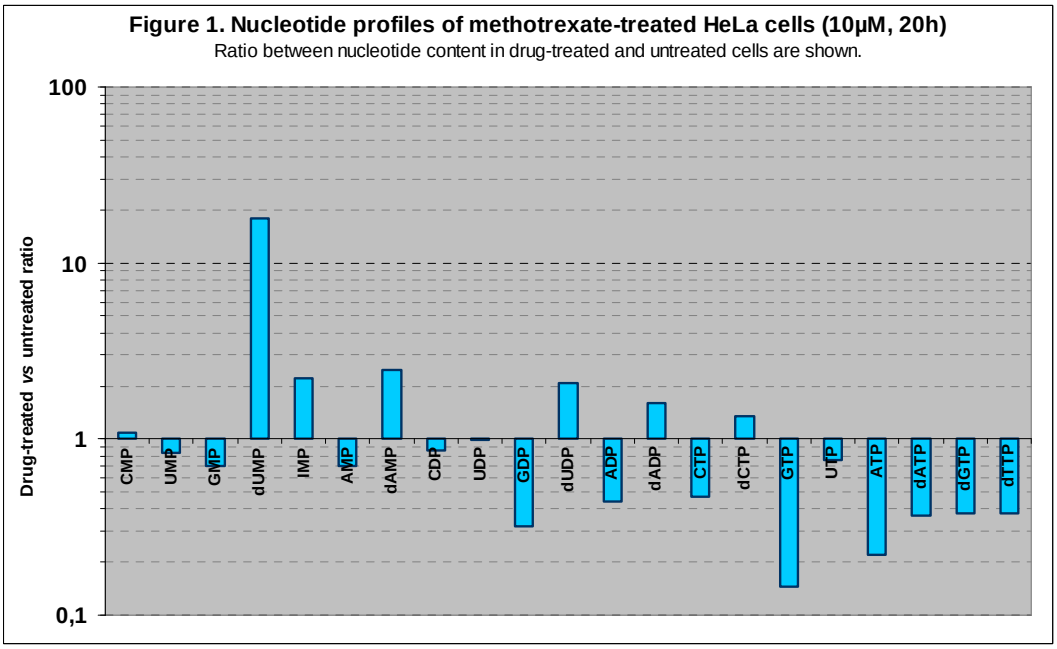


Figure 1. Nucleotide profiles of methotrexate-treated HeLa cells (10µM, 20h)
Ratio between nucleotide content in drug-treated and untreated cells are shown.



Methotrexate (MTX) is an immunosuppressive agent that has been in clinical use for over 50 years. Although originally introduced for chemotherapy in cancer and leukaemia, MTX was coincidentally found to have immunosuppressive properties and is currently used in treating rheumatoid arthritis[i]. MTX was first believed to be an inhibitor of the enzyme dihydrofolate reductase (DHFR), the enzyme required for reduction of dihydrofolate (FH_2) to tetrahydrolate (FH_4). However, as shown in the 80's, MTX is actually a prodrug which is polyglutamated and accumulated in cells[ii]. In contrast to unmodified MTX, its polyglutamated derivatives were found to be efficient inhibitors of the ninth folate-dependent step of purine synthesis catalysed by 5-amino-4-imidazolecarboxamide riboside 5'-monophosphate transformylase (AICAR-T)[iii] and the thymidylate synthase (TS)[iv].

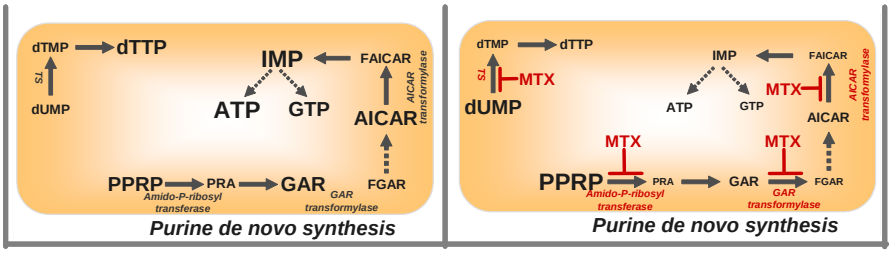


Figure 2. Effect of MTX on cellular pool of purines and pyrimidines.

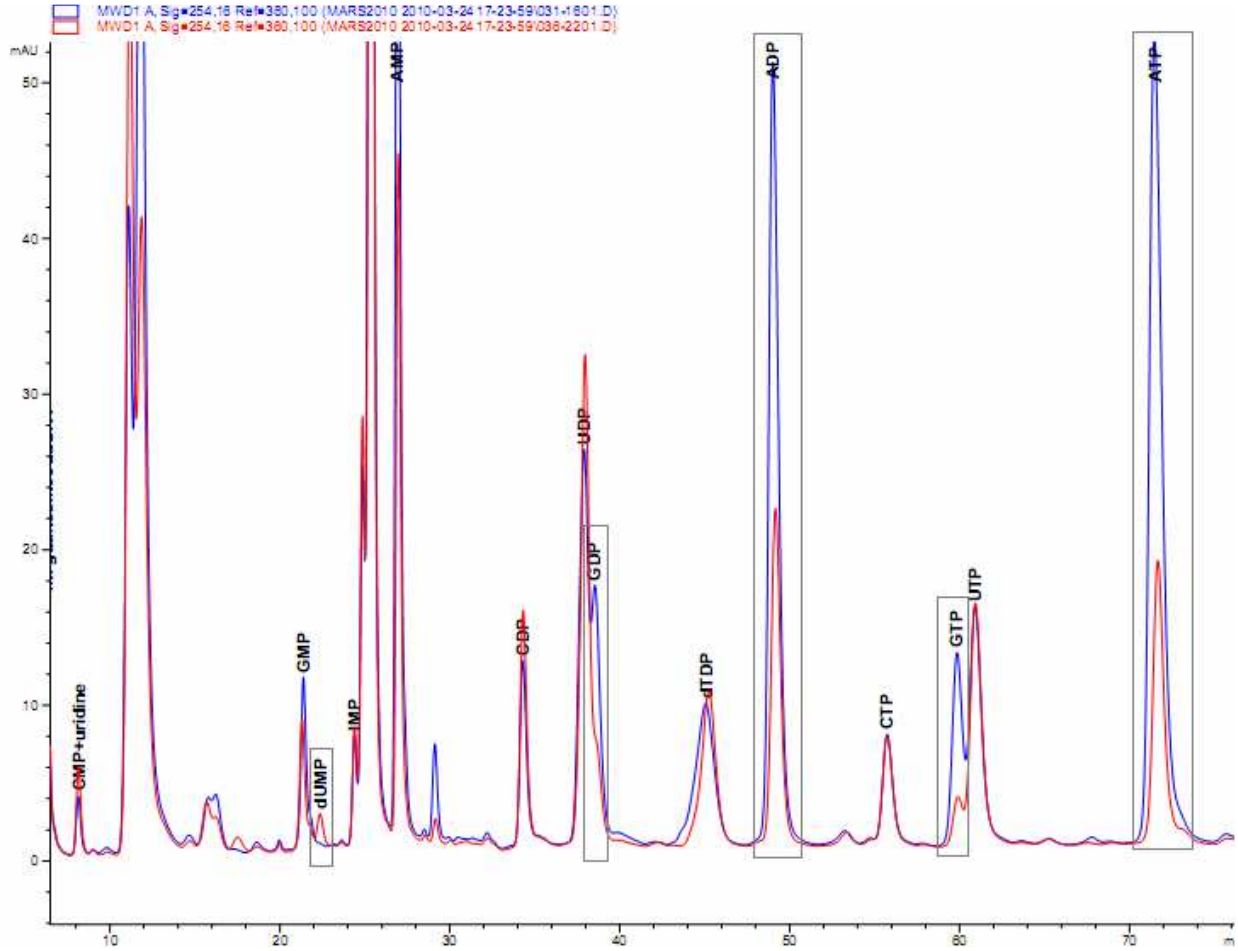


Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 10µM MTX (red) and DMSO (blue). The specific depletion in purine nucleotides, *e.g.* ATP, ADP, GTP and GDP, and accumulation of dUMP, as a result of TS inhibition, are framed in grey.

Results:

As illustrated by Figures 1 and 3, intracellular level of ATP, ADP, GTP and GDP is much lower in methotrexate-treated cells than in untreated control, while cellular contents of UTP and UDP are not affected. Another remarkable change concerns the accumulation of dUMP in methotrexate-treated HeLa cell and depletion of dTTP pool. All these results are in perfect agreement with previously published data[v] showing that MTX inhibits *de novo* synthesis of purine nucleotides through AICART enzyme and synthesis of thymidylate through thymidilate synthase.

Materials & Methods

Cells treatment: HeLa cells were grown in an atmosphere of humidified 5% CO_2 at 37°C in DMEM (PAA) medium supplemented with 2mM L-glutamine (Gibco/BRL), non essential amino acids (PAA), 10% heat-inactivated fetal bovine serum (BioWest) and streptomycin-penicillin (Sigma). Exponentially grown HeLa cells were seeded at ~$6x10^5$ cells per dish. After 48h of growth, the culture medium was replaced with fresh FCS-supplemented medium (10ml per Petri dish) followed by addition of 10µL of DMSO or DMSO-dissolved compounds. Six Petri dishes of cells per experiment were used to provide the nucleotide amount sufficient for UV-quantification of deoxynucleotides. At the end of a 7h-incubation, the medium was aspirated, cells monolayers washed twice with 5ml PBS, and used for nucleotides extraction.

Extraction of nucleotides and deoxynucleotides - Sample preparation: The nucleotides were extracted from cell monolayers by the addition of ice-cold 80% acetonitrile for 1h. The extracts were centrifuged to remove cellular debris and load on SAX column (100mg, Supelco) pre-conditioned with methanol, water and acetonitrile. Once the sample was completely effused, the cartridge was washed with 3ml 80% ACN and 3ml water and eluted with 1M KCl. The eluent was filtered through a 0.45µm filter membrane (Roth) and analyzed by HPLC.

Analytical system: 1) Agilent 1100 series liquid chromatograph fitted with binary pump G1312A, vacuum degasser G1322A, well-plate autosampler G1367A, thermostated column compartment G1316A and multiple wavelength and diode array detector G1315B. Run and data acquisition are controlled by Agilent ChemStation software.
2) Zorbax Extend-C18 4.6x150mm, 3.5µm particle size and corresponding guard column (Agilent).
5µl of cell extract were analyzed using Zorbax Extend-C18 column by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides with slight modifications as follows.
Peak identification of the different nucleoside mono-, di-, and triphosphates, was made from their characteristic UV absorption spectra and retention times compared with those of a mixture of standards (Sigma) run immediately before the cell extracts. The area of individual peaks was measured using ChemStation software (Agilent).

HPLC conditions: Nucleotides were analyzed by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides[vi] with slight modifications in pH and concentration of buffers adjusted to ensure adequate resolution of all nucleosides/nucleotides as follows: Buffer A: 20mM KH_2PO_4, 10mM tetrabutylammonium hydroxide pH 8.50; Buffer B: 100mM KH_2PO_4, 3mM tetrabutylammonium hydroxide, pH 3.0, 30% methanol. Flow rate: 1ml/min. Temperature kept constant at 21°C. Gradient was formed as follo ws: 15 min at 100% buffer A; 5 min up to 90% buffer A, 5 min up to 70% buffer A; 15 min up to 63% buffer A, 15 min up to 55% buffer A, 20 min up to 45% buffer A, 10 min up to 25% buffer A, 10 min up to 0% buffer A. The spectra were recorded at 254 and 280nm.

HPLC calibration: The calibration was performed with the following standards: dUMP, dUDP, dUTP, dCDP, dCMP, dCDP, dCTP, dTMP, dTTP. dGDP and dGTP were not separated from unknown major peak and were not quantified. The standards prepared in Buffer A or those mixed with cell extracts were run immediately before and after series of samples. The data were used for calculation of retention times (Rf) and absorbance at 254nm and 280nm (254/280 ratio), specific for each nucleotide.

Peak identification and quantification: 5µl of cell extract were injected and nucleotides were separated as described before. Assignment of peak of the different deoxyribonucleosides and ribonucleosides mono-, di-, and triphosphate was done by comparing both retention times and characteristic UV absorption spectra (254/280 ratio) with those of standards. The area of individual peaks was measured using ChemStation software (Agilent).

Quality control: The experiments are done in duplicates and relative standard deviation (RSD) is usually less than 12%.

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