

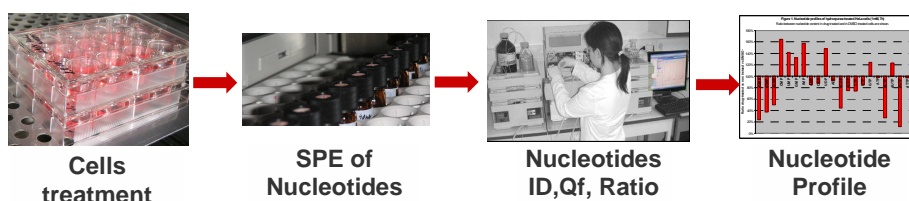
RNR inhibition - Whole Cell Assay

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-paired 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)

Nucleotide profiles of hydroxyurea-treated HeLa cells (1mM, 20h)

Ratio between nucleotide content in drug-treated and untreated cells are shown.

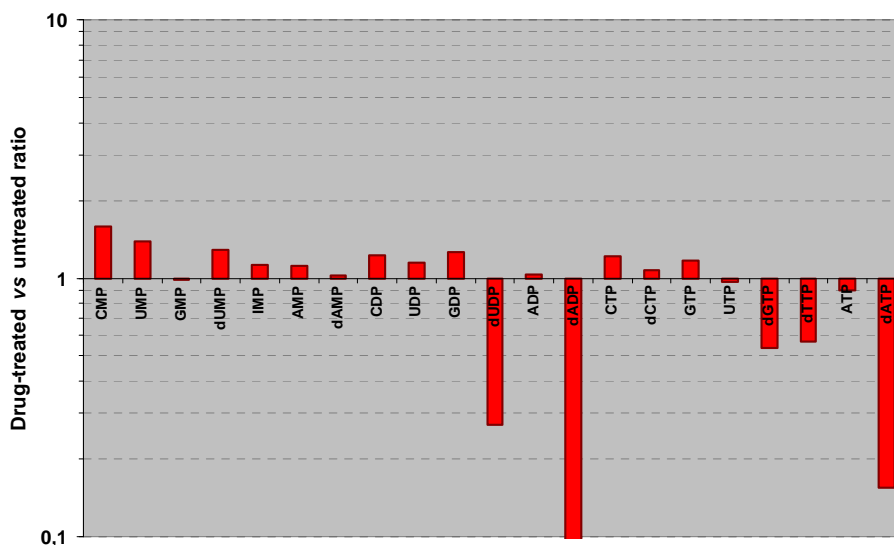


Figure 1: Nucleotide profiles of hydroxyurea-treated HeLa cells

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}.

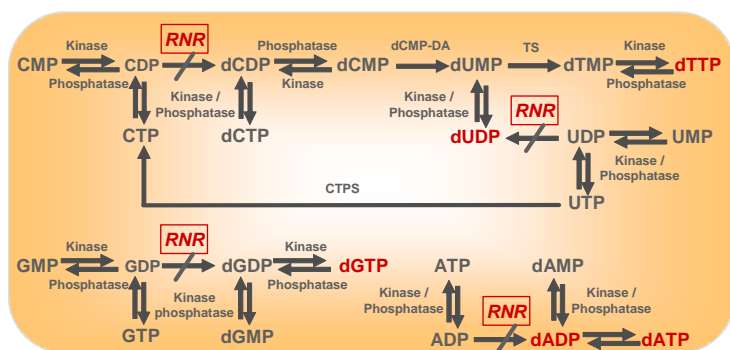


Figure 2. Effect of hydroxyurea on cellular pool of deoxynucleotides. The depleted nucleotides are shown in red. Ribonucleotide reductase (RNR), a recognized target of hydroxyurea, is framed in red.

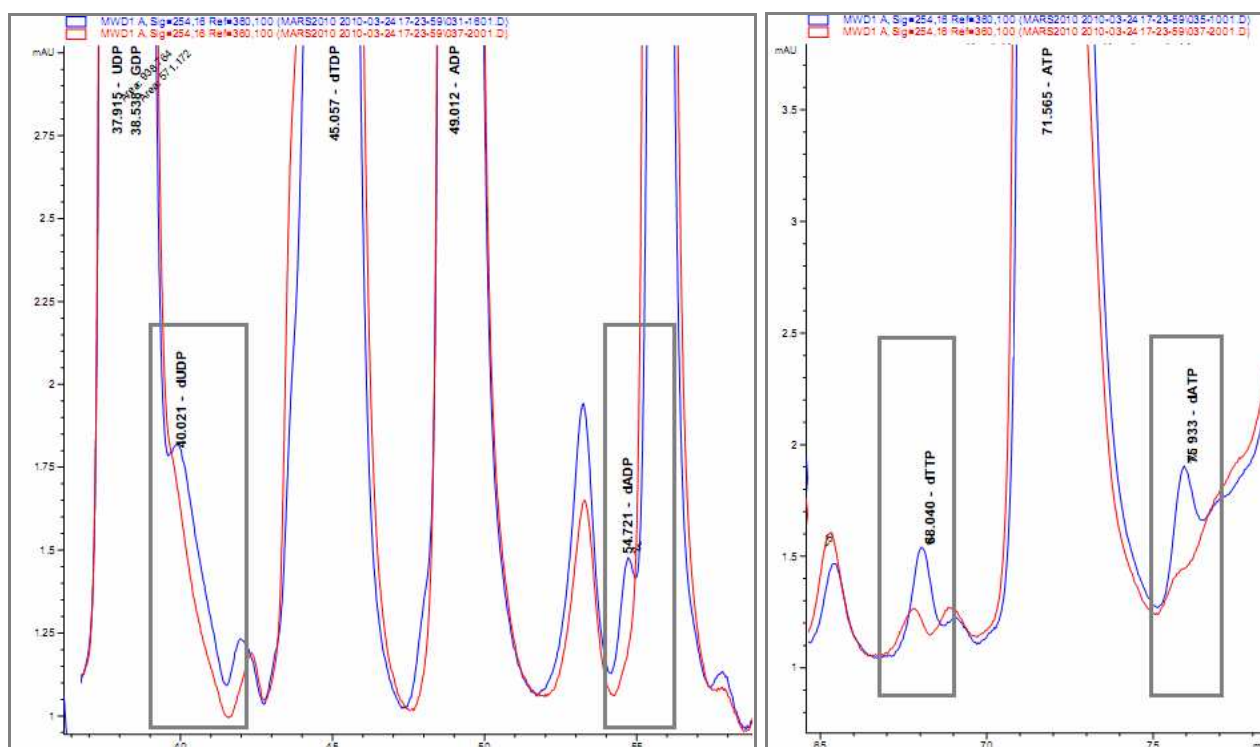


Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 1mM hydroxyurea (red) and DMSO (blue). Focus on depletion in dUDP and dADP is shown on left and in dTTP and dATP on right.

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)

Nucleotide profiles of Gmc-treated HeLa cells (37µM, 20h)
Ratio between nucleotide content in drug-treated and untreated cells are shown.

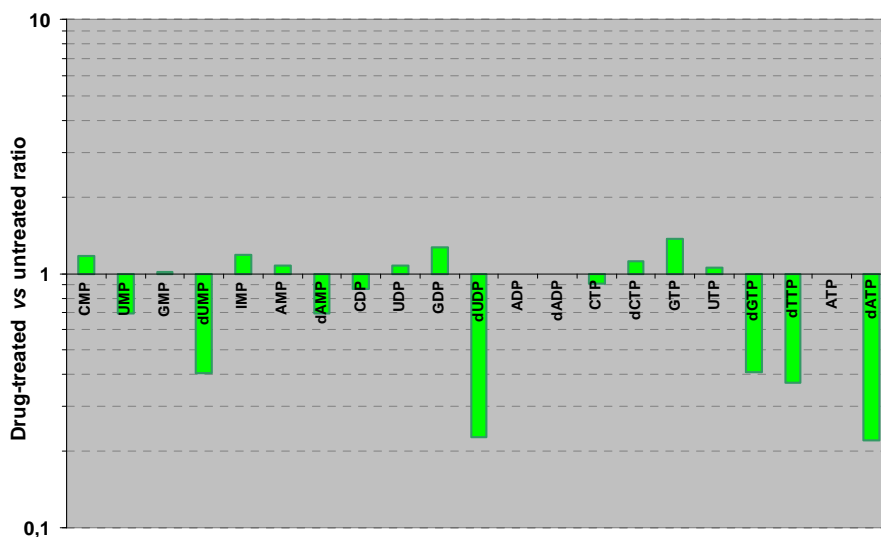


Figure 4: Nucleotide profiles of gemcitabine-treated HeLa cells

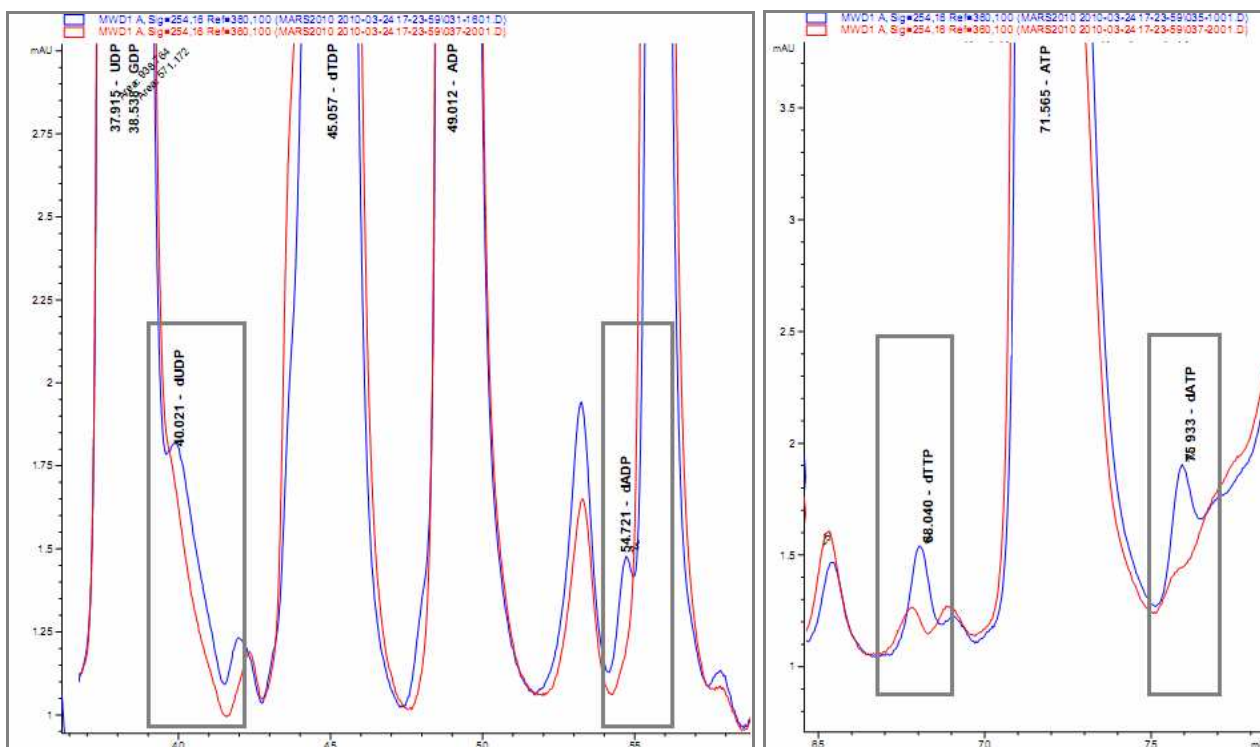


Figure 3. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 1mM hydroxyurea (red) and DMSO (blue). Focus on depletion in dUDP and dADP is shown on left and in dTTP and dATP on right.

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 data¹, but may also reflect the decrease in cellular dUDP, a source of dUMP.

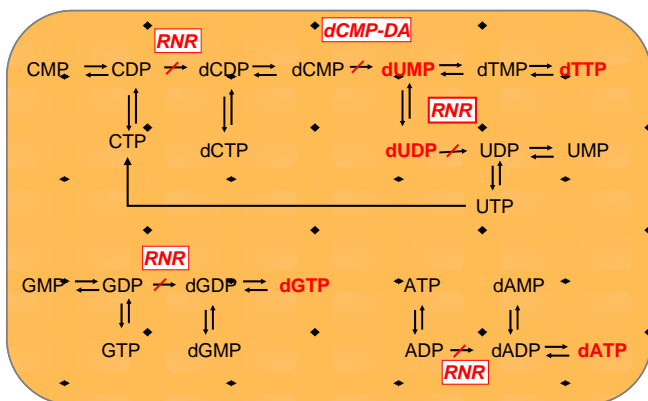


Figure 5. Effect of gemcitabine on cellular pool of nucleotides and deoxynucleotides. The depleted (<50% of control) nucleotides are shown in red. Ribonucleotide reductase (RNR) and dCMP-deaminase (dCMP-DA), recognized targets of gemcitabine, are framed in red.

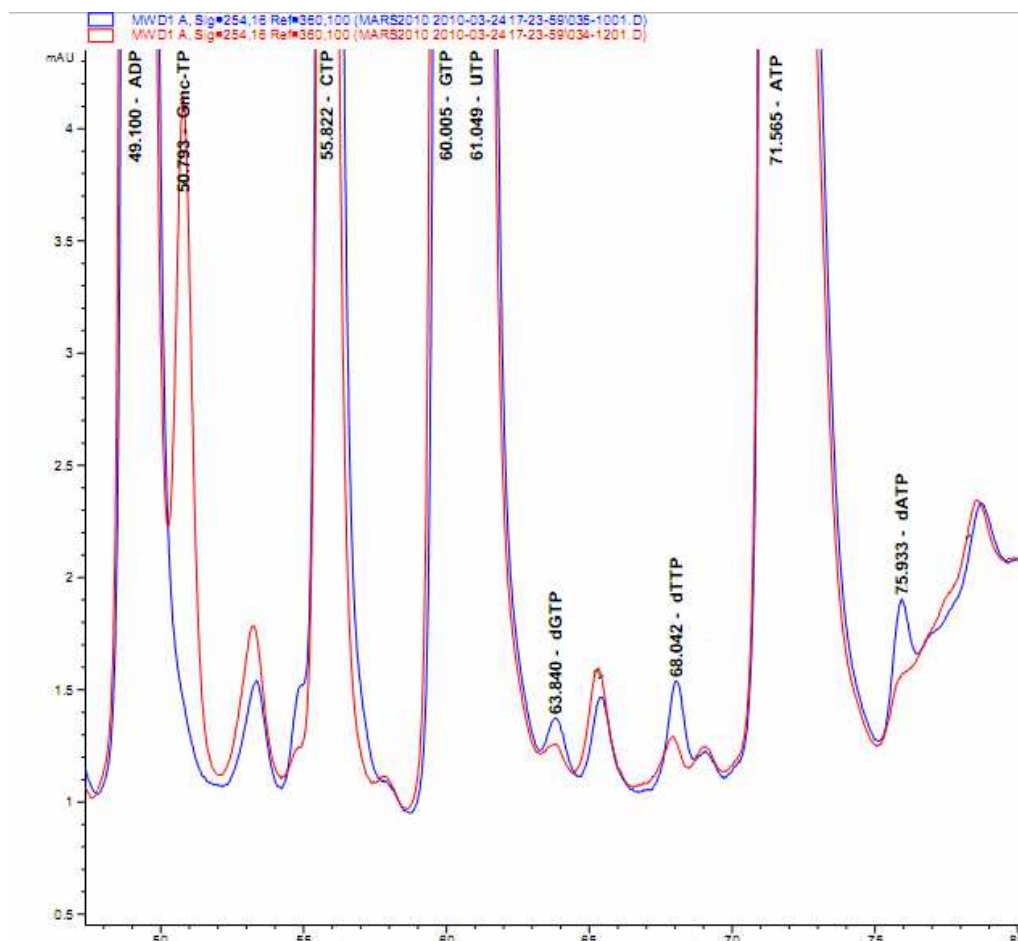


Figure 6. Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 37µM gemcitabine (blue) and DMSO (red) illustrating depletion in dNTP.

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% CO₂ 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 ~6x10⁵ 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 nucleotides¹ 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 KH₂PO₄, 10mM tetrabutylammonium hydroxide pH 8.50; Buffer B: 100mM KH₂PO₄, 3mM tetrabutylammonium hydroxide, pH 3.0, 30% methanol. Flow rate: 1ml/min. Temperature constantly kept at 21°C. 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 (R_f) 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