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.

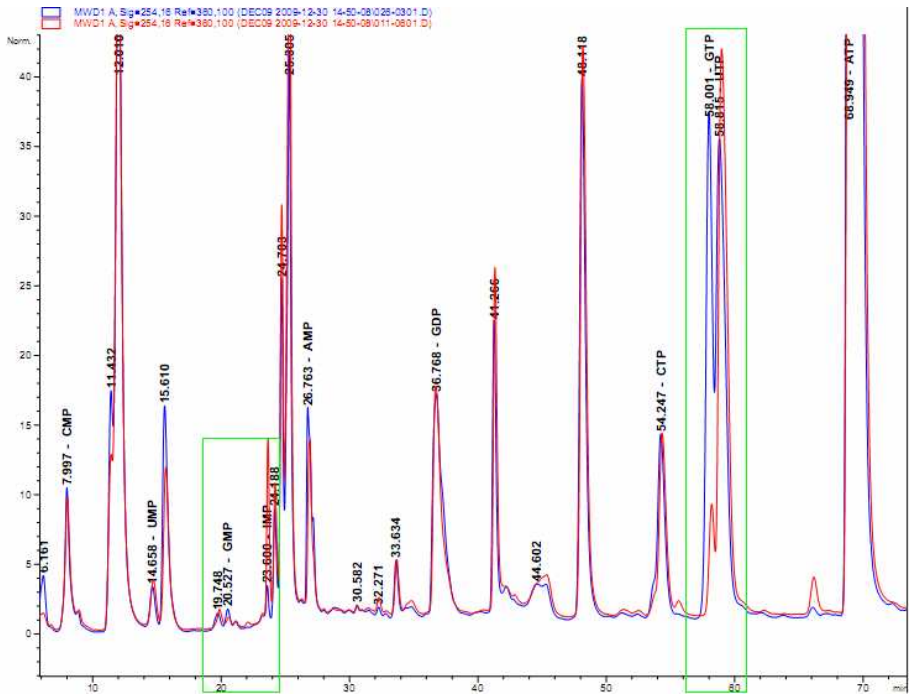
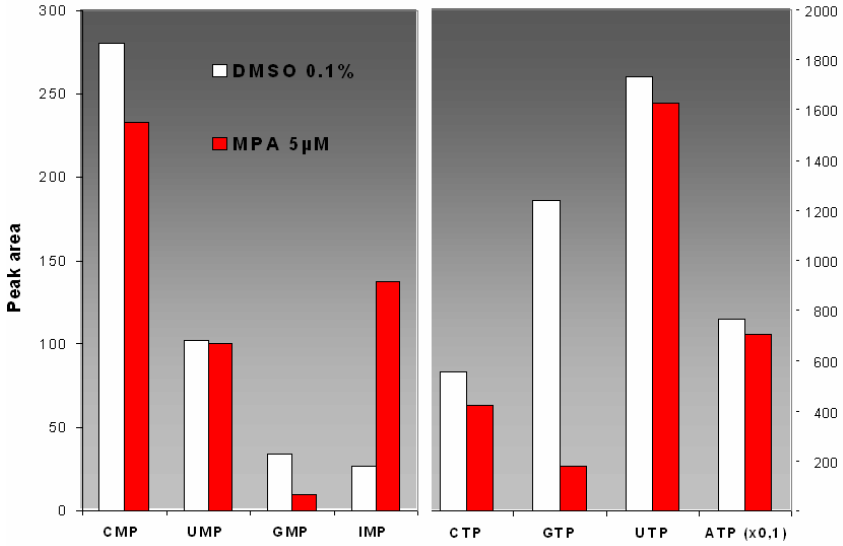


Figure 1. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 5µM MPA (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.

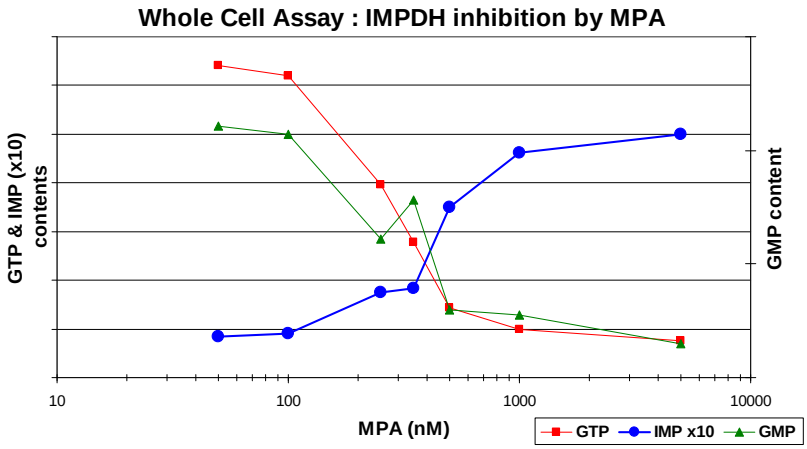


Concentration of nucleotides mono- and tri-phosphate in MPA- and DMSO-treated cells (measures as peak area, AU)

	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.

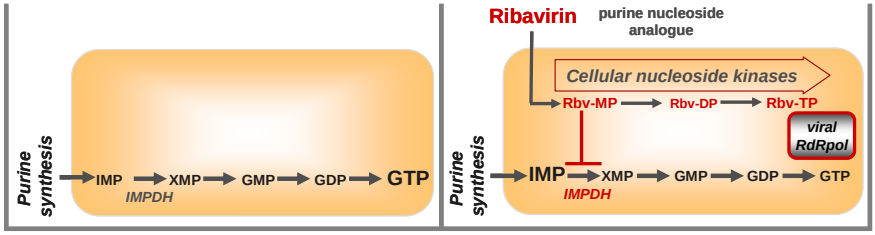


Figure 3. Modifications in cell-pool of nucleotides in Ribavirin-treated cells

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.

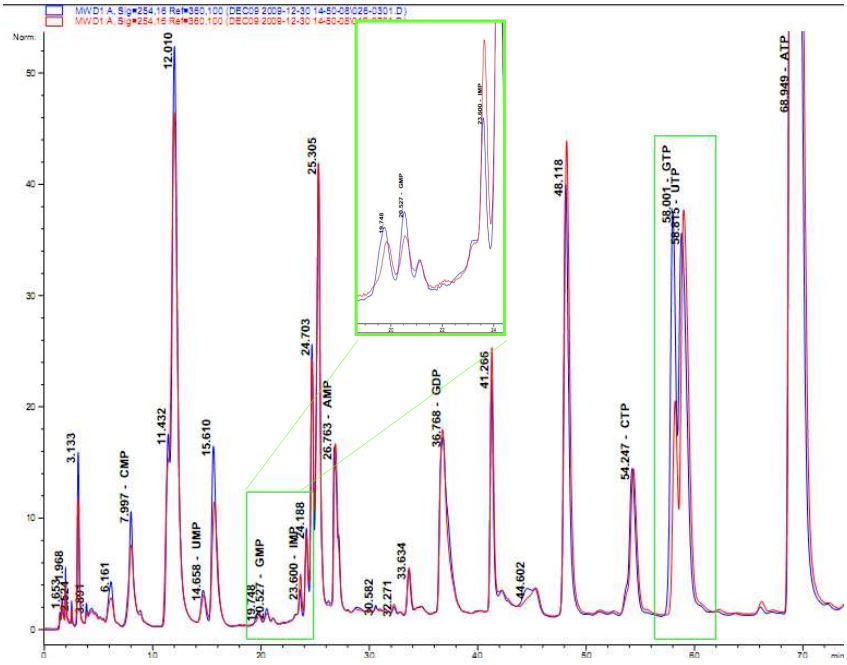
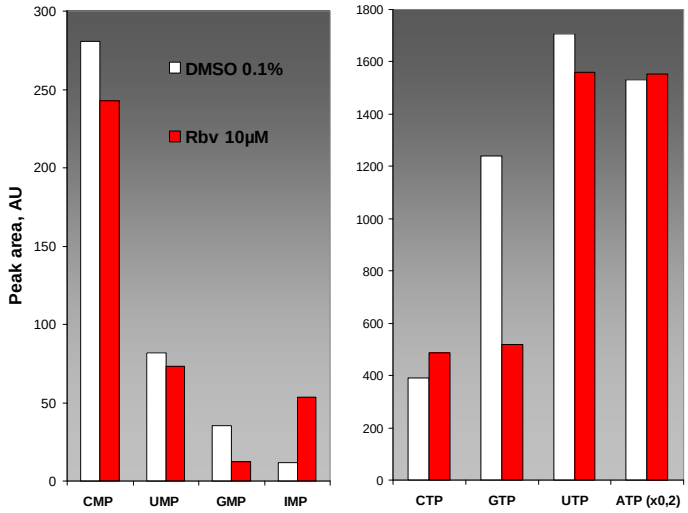


Figure 4. Superposition of HPLC spectra of nucleotide extracts of Huh-7 cells incubated for 48h in the presence of 10µM Rbv (red) and 0.125% DMSO (blue). The changes in cellular GTP, GMP and IMP are framed in green.



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