Bio-analytical assays for evaluation of nucleotide biosynthesis inhibition in cultured cell model

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ABSTRACT

Numerous nucleoside analogues are currently used to treat viral infections and cancer. They are usually designed either to be effectively incorporated by polymerase and to act as chain-terminators or to directly inhibit target polymerase. However, once entered the cells, the analogues of nucleosides may also inhibit biosynthesis of cellular nucleotides acting also as anti-metabolites. The changes, by a given nucleoside analogue, of cellular pool of nucleotides can be the reasons of its cytostatic or cytotoxic effect. However, if depleted cellular nucleotide is also a natural competitor of nucleoside analogue, this may result in its increased incorporation and higher efficacy, as observed with ribavirin and gemcitabine.

To provide a new investigation tool for early detection of anti-metabolite effect of new nucleoside analogues, we have developed a cell-based bio-analytical assay where up to 31 cellular nucleosides, ribo- and deoxyribonucleotides (mono-, di-, triphosphate) and nucleotide co-factors are extracted from cultured cells, separated by ion-pairing chromatography and quantified. To validate this tool, we have characterized nucleotide composition of Huh-7 cells (i) under varying culture conditions, (ii) in the presence of known antimetabolites, such as ribavirin, mycophenolic acid (MPA), gemcitabine, methotrexate, hydroxyurea; (iii) in the presence of exogenous purine bases and nucleosides.

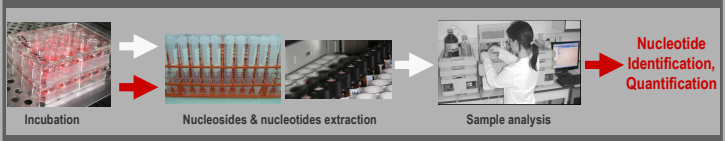
Introduction

Purine and pyrimidine nucleotides play crucial roles in major cell functions. In addition to being basic building blocks of nucleic acids and coenzymes (NAD, Co-A) nucleosides and nucleotides are also involved in numerous cellular processes, such as energy metabolism (ATP, GTP), phospholipids biosynthesis (CDP-glycerol), protein glycosylation (UDP-sugars), cell signalling (cAMP, cGMP), methylation (SAM), neurotransmission (adenosine). This remarkable diversity of function emphasizes the fundamental importance of nucleotide for cells. Mammalian cells maintain cellular level of purine and pyrimidine nucleotides through two separate metabolic pathways: by de novo synthesis or by salvage of extracellular purine bases and nucleosides. The enzymes involved in nucleotide biosynthesis are differently expressed in different cell lines and their activity depend also on growth conditions and culture media [1, 2]. In addition, tumor cells, major source of cultured cell line, are known to have higher activity of nucleotide anabolic pathway over catabolic pathway. Elevated rates of nucleotides biosynthesis in tumor cells is achieved through (i) increased synthesis of nucleic acid ribose via non-oxidative branch of pentose phosphate pathway [3]; and (ii) up-regulation of the genes in human purine and pyrimidine biosynthesis pathway by c-Myc oncoprotein [4].

Human hepatoma cell line Huh-7 was found to efficiently support replication of HCV-derived replicon [5, 6]. Huh-derived particular cell clones, designated Huh7.5 and Huh7.5.1 are highly permissive for HCV infection and support higher levels of HCV RNA replication as compared to naive Huh-7 cells [7, 8]. Huh-7 and its clones are therefore widely used for the search of new antiviral molecules, including new nucleoside analogues, against HCV. However, little is known about nucleoside transport and nucleotide biosynthesis in these cells that are known to have preserved certain morphologic and biochemical characteristics of hepatocytes. The aim of this study was to characterize the dynamic of nucleotide pool of Huh-7 cells in various culture conditions as well as to measure anti-metabolic impact of ribavirin, gemcitabine and other known anti-metabolites in this cell line.

Materials and Methods

Huh-7 cells were grown in an atmosphere of humidified 5% CO_2 at 37°C in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum. Exponentially grown cells were seeded at ~$6x10^5$ cells per 10-cm dish, and DMSO-dissolved compounds were added to medium for indicated time. At the end of incubation, the medium was aspirated, cells monolayers washed and used for nucleotides extraction by SPE using pre-conditioned SAX columns. The eluents were analyzed by ion-pairing HPLC method reported previously for the simultaneous separation and quantification of bases, nucleosides and nucleotides [7] with slight modifications. Peak identification of bases, different nucleosides and nucleoside mono-, di-, and triphosphates, was made based on Rf of standards mixed with cell extracts and run immediately before and after series of samples and absorbance 254nm/280nm ratio. The area of individual peaks was measured using ChemStation software (Agilent).



We have used our bio-analytical method to characterize nucleotide content of Huh7 cells and to study the effect of ribavirin (Rbv) and mycophenolic acid (MPA), known inhibitors of IMPDH, key enzyme of GTP biosynthesis, on nucleotide pool.

Results and Discussion

IMPDH inhibition

Incubation of Huh-7 cells with MPA (5µM), a known inhibitor of cellular IMPDH (E.C. 1.1.1.205), enzyme that catalyzes the pivotal step in guanine nucleotide biosynthesis: the conversion of IMP to XMP, results in dramatic depletion of cellular GTP (Fig. 1). Huh-7 cells provide therefore a valuable model for evaluation of IMPDH inhibitors.

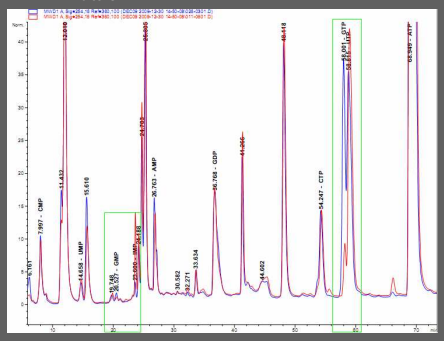


Fig. 1: Superposition of HPLC spectra of nucleotide extracts of Huh 7-5 cells incubated for 48h in the presence of 5µM MPA (red) and 0.125% DMSO (blue). Changes in cellular GTP, GMP and IMP are framed in green.

Table 1. Concentration of nucleotide in MPA- treated cells (peak area, AU)

Rf		DMSO 0.1%	MPA 5µM
7.9	CMP	280,2	233,2
14.6	UMP	102,2	100,2
20.5	GMP	33,84	9.6
23.6	IMP	26,98	137,19
26.9	AMP	396,64	273,2
54.2	CTP	554	424,8
58.0	GTP	1237	182
58.8	UTP	1734,8	1627
68.9	ATP	7665	7057,4

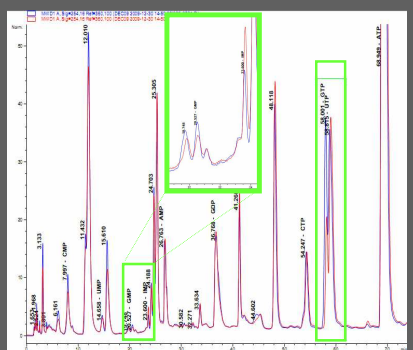
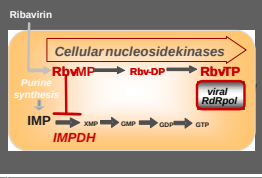


Fig. 2: Superposition of HPLC spectra of nucleotides extracted from Huh-7 cells treated with ribavirine (10µM) pendant 48h (red) and non treated cells (blue). Specific changes in GTP, GMP and IMP are shown in frame.

Incubation of Huh-7 cells with Rbv (10µM), also leads to depletion of GTP pool consistently with known effect of Rbv-MP on human IMPDH. Even if direct relationship between ribavirin antiviral action and IMPDH inhibition by Rbv-MP has not been demonstrated, the depletion of cellular GTP might result in increased frequency of Rbv-TP incorporation by viral polymerase due to lower intracellular concentration of its natural competitor.



Unexpectedly, cellular GTP was found to be the most affected by variations in cell culture conditions, in comparison with other NTPs. Nevertheless, both Rbv and MPA provoke a specific GTP depletion in Huh-7 cells at all conditions tested.

	Nucleotide content, % of total NTP			
	Cond.1	Cond.2	Cond.4	Cond.7
ATP	79.9%	71.0%	69.1%	73.0%
UTP	16.2%	16.5%	15.1%	18.3%
GTP	1.1%	7.8%	10.8%	3.8%
CTP	2.8%	4.7%	5.0%	4.8%

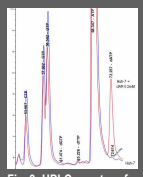
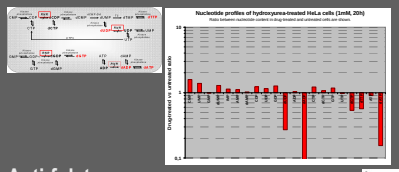


Fig. 3: HPLC spectra of extracts of Huh-7 cells incubated in the presence of 200µM dAR (2h).

The same approach was applied to study the uptake of extracellular nucleosides and their subsequent metabolism in Huh-7 cells. Of all bases and nucleosides studied, inosine and guanosine were found to be most efficiently transported by Huh-7 cells with following order IR, GR > AR, dGR, Hx, > dIR, dAR, A. Rapid deamination of adenosine and deoxyadenosine indicates a high level of ADA activity in Huh-7 cells, whereas efficient formation of dATP from dAR leading to 300-fold expansion of dATP pool, (Fig. 3) implies that Huh-7 cells have a very high level of corresponding nucleoside kinase(s).

RNR inhibition

For further validation of our bio-analytical method, we studied the effect of hydroxyurea and methotrexate (MTX). Hydroxyurea is a known inhibitor of class I ribo-nucleoside diphosphate reductase (RNR) on pool of nucleotides and deoxynucleotides in HeLa cells. As illustrated by Fig. 4, hydroxyurea treatment induces in HeLa cells specific depletion of deoxyadenosine triphosphate and significant loss of dADP, dUDP and dTTP, which is consistent with previously published data.



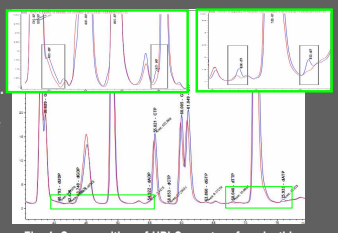


Fig. 4: Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 1mM hydroxyurea (red) and DMSO (blue). Focus on depletion in dUDP and dADP is shown on left and and dTTP, dATP on right

Anti-folates

As illustrated by Fig. 5, MTX specifically inhibits intracellular level of ATP, ADP, GTP and GDP in HeLa cells, while cellular contents of UTP and UDP are not affected. Another remarkable change concerns the accumulation of dUMP in MTX-treated HeLa cell and depletion of dTTP pool. All these results are in perfect agreement with previously published data showing that MTX inhibits de novo synthesis of purine nucleotides through AICART enzyme and synthesis of thymidylate through thymidylate synthase (TS).

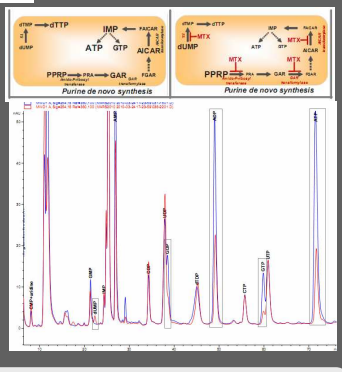


Fig. 5: Superposition of HPLC spectra of nucleotides extracted from HeLa cells treated with 10µM MTX (red) and DMSO (blue). The specific depletion in purine nucleotides, e.g. ATP, ADP, GTP and GDP, and accumulation of dUMP, as a result of TS inhibition, are framed in grey.

Conclusion

Our bio-analytical assay provides a highly informative and validated tool for evaluation of nucleotide and deoxynucleotide biosynthesis in cell culture model.

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