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Nucleoside Metabolism Explorer

Development of *in vitro* and cell-based assays for assessing nucleotide biosynthesis inhibition

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Background Numerous nucleoside analogues (NAs) are currently used to treat viral hepatitis. They are usually designed to inhibit one viral target. This remains in contrast with the observation that ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), 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 by the 5'-triphosphate metabolite of ribavirin, (iii) induction of error catastrophe as a result of accumulation of mutations in the viral genome. Even if no direct relationship between ribavirin antiviral action and IMPDH inhibition has been demonstrated, the depletion of cellular GTP should result in an increased frequency of ribavirin triphosphate incorporation by viral polymerase due to lower intracellular concentration of its natural competitor.

Aims Relation between therapeutic potential of a nucleoside analogue and its anti-metabolite action remains difficult to demonstrate mainly because of the lack of investigation tools. The purpose of this study was to develop a range of assays to reveal nucleotide biosynthesis inhibition.

Methods and Results For a rapid evaluation of new nucleoside analogues as IMPDH inhibitors, we have developed an *in vitro* enzymatic assay where the synthesis of monophosphorylated form of nucleoside analogue (NA-MP) is provided by cloned human nucleoside kinases, and NA-MP is immediately tested for inhibition of human recombinant IMPDH. This assay has been validated with nucleoside analogues ribavirin and mizoribine.

We have also developed original cell-based analytical approach in which 27 cellular ribo- and deoxyribonucleotides are extracted from cultured cells, separated by ion-pairing chromatography and quantified. This cellular assay, validated with several NA (ribavirin, aracytidine, gemcitabine) and known anti-metabolites (mycophenolic acid, leflunomide, hydroxyurea), provides a powerful tool for studying the effect of new nucleoside analogues on whole spectra of cellular purine and pyrimidine deoxy- and ribonucleotides. In addition, in regards with new antiviral molecules identified in HCV cell culture systems, our cellular assay allows to distinguish the molecules that directly acts on the viral proteins from others that inhibits the cell nucleotide biosynthesis.

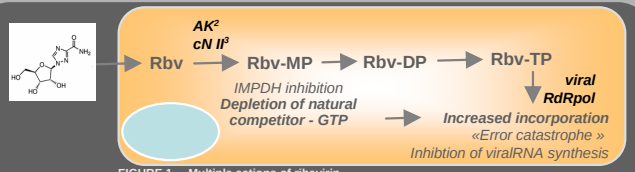


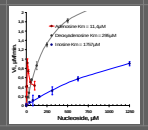
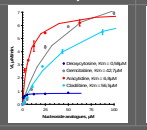
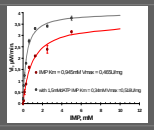
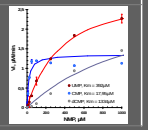
FIGURE 1. Multiple actions of ribavirin.

Ribavirin (1-b-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, ref. 1) is a purine nucleoside analogue with broad spectrum antiviral activity. Since the 1970's, it is known that the initial step of ribavirin phosphorylation is provided by adenosine kinase (2). Recently it has been demonstrated that cytosolic 5'-nucleotidase II can also phosphorylate ribavirin, that could contribute to the development of ribavirin-induced haemolytic anemia in vivo (3). Monophosphorylated form of Rbv was shown to inhibit cellular IMPDH, whereas tri-phosphorylated form, after being incorporated by viral RNA-dependent RNA-polymerases, induces "error catastrophe" and inhibits RNA synthesis.

Enzymatic assays for the characterization of phosphorylation properties of new nucleoside analogues

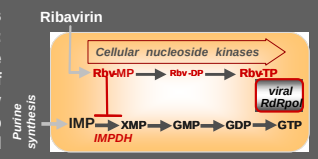
The therapeutic efficacy of nucleoside analogues is highly dependent on their intracellular phosphorylation. Nucleoside phosphorylation assays focused on cloned human recombinant nucleoside kinases (dCK, AK, YMPK, cNII) have been developed and validated with known NAs (Table 1). Vmax and Km values of human recombinant nucleoside kinase were determined for natural substrates and nucleoside analogues (*e.g.* ribavirine, araC, gemcitabine, etc). The results obtained are highly similar to previously published data. These assays now allow the characterization of substrate properties (Km and Vmax) of new nucleoside analogues in comparison with those of known nucleoside analogues.

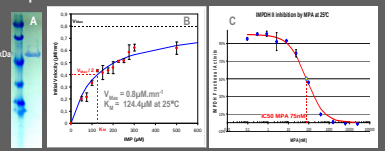
Table 1. Cloned human recombinant nucleoside kinases and their substrates.

	Human recombinant Adenosine kinase EC 2.7.1.20	Human recombinant Deoxycytidine kinase EC 2.7.1.74	Human recombinant cytosolic 5'-nucleotidase / phosphotransferase II EC 3.1.3.5	Human recombinant UMP-CMP kinase EC 2.7.4.14
	GenBank U50196 100% identity 39kDa	GenBank P27707 100% identity 33kDa	GenBank P49902 100% identity 65kDa	GenBank FLJ93091 99% identity 27KDa
Natural substrates	Adenosine Inosine	Deoxyadenosine Deoxyguanosine Deoxycytidine Cytidine	Deoxyinosine Inosine	dCMP CMP UMP
Nucleoside analogue substrates	Ribavirin Tubercidin Mizoribin	Cladribine, fludarabine Gemcitabine, Lamivudine, Aracytidine Fluorodeoxyuridine	Dideoxyinosine Ribavirin Acyclovir	dFdCMP 3TCMP araCMP Adefovir (PMEA)
Substrate properties				

Enzymatic characterization of human recombinant IMPDH II

IMP Dehydrogenase (IMPDH, E.C. 1.1.1.205) catalyzes the pivotal step in guanine nucleotide biosynthesis: the conversion of IMP to XMP. Blocking the conversion of IMP to XMP, Rbv leads to depletion of GTP pools, that results in increased frequency incorporation of Rbv-TP by viral polymerase due to lower intracellular concentration of its natural competitor.





Enzymatic assay with cloned human IMPDH II was developed and validated by measuring Vmax and Km value for IMP and IC50 value for MPA.

FIGURE 2: A. The cDNA encoding human IMPDH II was cloned by RT-PCR amplification of RNA from human hepatocarcinoma Huh7. The sequence of cloned IMPDH II (P22168) was confirmed by sequencing (100% identity). The IMPDH II protein was overexpressed in E.coli and purified. B. Km and Vmax values of recombinant human IMPDH II were measured spectrophotometrically by monitoring the formation of NADH at 340nm C. IC50 value of mycophenolic acid (MPA) was measured at 180µM NAD and 100µM IMP.

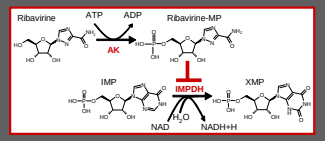
References

[1] D.G. Streeter, J. T. Witkowski, G.P. Khare, R.W. Sidwell,r.j. Bauer, R.K. Robins, L. N. Simon (1973) Mechanism of Action of 1-i3-D-Ribofuranosyl-1,2,4-Triazole-3-Carboxamide (Virazole), A New Broad-Spectrum Antiviral Agent Proc. Nat. Acad. Sci. USA Vol. 70, No. 4, pp. 1174-1178

[2] R. C. Willis, D. A. Carson, and J. E. Seegmiller (1973) Adenosine Kinase Initiates the Major Route of Ribavirin Activation in a Cultured Human Cell Line. PNAS USA, 75: 3042-3044.

[3] Wu JZ, Larson G, Walker H, Shim JH, Hong Z. Phosphorylation of ribavirin and viramidine by adenosine kinase and cytosolic 5'-nucleotidase II: Implications for ribavirin metabolism in erythrocytes. (2005) Antimicrob Agents Chemother. 49(6):2164-71

Coupled nucleoside kinase -IMPDH II assay for rapid evaluation of monophosphate forms of nucleoside analogues



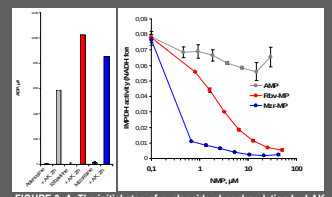


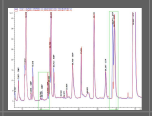
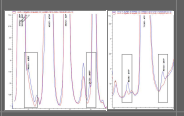
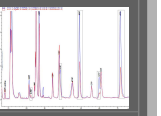
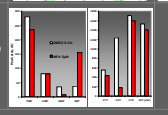
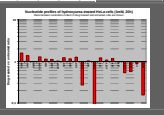
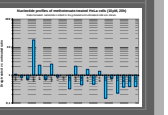
FIGURE 3: A. The initial step of nucleoside phosphorylation by hAK was followed in spectrophotometric LDH-PK system. The concentration of monophosphorylated nucleoside was quantified as ADP formed. B. The IMPDH activity was spectrophotometrically determined at 25°C by monitoring NADH formation.

Nucleoside analogues need to be phosphorylated to inhibit IMPDH. For rapid evaluation of NA-monophosphate as IMPDH inhibitors, we have developed an *in vitro* enzymatic assay based on cloned human nucleoside kinases and human IMPDH II.

In this assay, the monophosphorylation step of nucleoside analogue is provided by specific nucleoside kinases; the enzymatically produced NA-monophoshate is then directly tested for IMPDH inhibition without puriifcation. This combined test has been validated with Rbv and mizoribine.

Whole cell bio-analytic assays: nucleotide profiling

To allow the study of whole spectra of purine and pyrimidine ribo- and deoxyribonucleotides in drug-treated cultured cells, we have developed whole cell bio-analytic assay. More than 27 (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 both with anti-viral and anti-cancer nucleoside analogues and known anti-metabolites.

	IMPDH inhibition Whole Cell Assay	RNR inhibition Whole Cell Assay	Multi-targeted antifolates Whole Cell Assay
Inhibitors	Ribavirin Mycophenolic Acid (MPA)	Hydroxyurea Gemcitabine	Methotrexate
Spectra			
Nucleotide Profiles			

In conclusion, we have developed a range of new tools that allow

1) to characterize *in vitro* phosphorylation properties of new nucleoside analogues, using recombinant human nucleoside kinases

2) to characterize IMPDH inhibition by mono-phosphorylated form of NAs

2) to study anti-metabolite effect of new anti-viral molecules and to distinguish "true" antiviral molecules from inhibitors of cell nucleotide biosynthesis

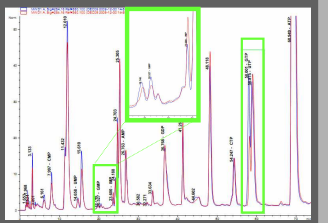


FIGURE 4: A. Superposition of HPLC spectra of nucleotides extracted from Huh-7 cells treated with ribavirine (10µM) pendant 48h (red) and non treated cells (blue). Specific changes in GTP, GMP and IMP are shown in frame.

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