Targeting the enzymes of nucleoside biosynthesis provides a way for discovery of novel 'dual specificity' inhibitors of hepatitis C virus

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ABSTRACT

Numerous nucleoside analogues (NA) are currently used to treat HBV, HIV and herpes simplex viral infections. They are usually designed to inhibit one viral target, the viral polymerase. However, traditional single-drug, single-target paradigm in drug discovery is not always the most successful and there are numerous examples of drugs that mediate their effects through multiple targets. Ribavirin, a broad-spectrum antiviral nucleoside analogue used to treat hepatitis C (HCV) infection, is a classic example of such a drug – its monophosphorylated form inhibits cellular IMPDH, whereas tri-phosphorylated form, after being incorporated by viral RNA-dependent RNA-polymerases, induces "error catastrophe" and inhibits RNA synthesis. For rapid evaluation of monophosphate forms of nucleoside analogues as IMPDH inhibitors, we have developed an *in vitro* enzymatic assay consisting of cloned human nucleoside kinases and cloned human IMPDH type 2. In this assay the monophosphorylation of nucleoside analogue is provided by specific nucleoside kinases, and enzymatically produced NA-monophoshate is immediately tested for IMPDH inhibition. This combined test has been validated with nucleoside analogues ribavirin and mizoribine, both known inhitors of human IMPDH.

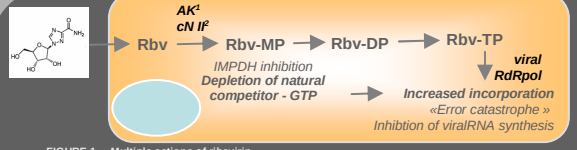

FIGURE 1. Multiple actions of ribavirin.

Ribavirin,(1-b-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) a purine nucleoside analogue with broad spectrum antiviral activity, is a classic example of multitarget drug. Various mechanisms of action have been suggested to be responsible for the antiviral activity of ribavirin:
(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 (some of them lethal) in the viral genome.

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, IMPDH inhibitors lead to depletion of the guanylate pools. The therapeutic consequences of IMPDH inhibition vary for different analogues - ribavirin is a broad spectrum antiviral and mizoribine is an immunosuppressor. 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 ribavirin triphosphate incorporation by viral polymerase due to lower intracellular concentration of its natural competitor.

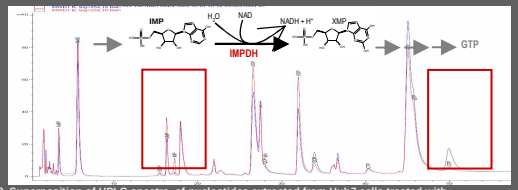

FIGURE 2. Superposition of HPLC spectra of nucleotides extracted from Huh7 cells treated with ribavirine (200µM) pendant 48h (red) and non treated cells (blue).

Since 1970s it is known that the initial step of ribavirin phosphorylation is provided by adenosine kinase (3). 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 (4). Both human recombinant adenosine kinase and cytosolic nucleotidase II were cloned to develop nucleoside phosphorylation assays.

Enzymatic characterization of human recombinant adenosine kinase

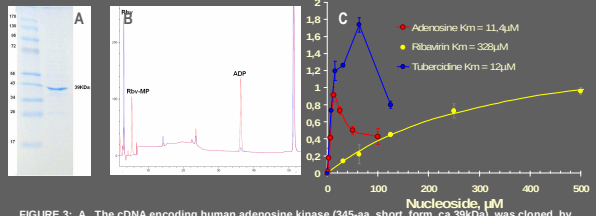

FIGURE 3: A. The cDNA encoding human adenosine kinase (345-aa short form, ca.39kDa) was cloned by RT-PCR amplification of mRNA extracted from human hepatoma cells and expressed in *E.coli*. The sequence of the cloned AK (GenBank accession number U50196) was confirmed by DNA sequencing (100% identity). The enzyme purity is controlled by 12% SDS-PAGE.
B. The phosphorylation of ribavirin by adenosine kinase was confirmed by HPLC analysis as illustrated by ribavirine-MP formation (red) from ribavirine (blue).
C. Enzymatic activity of adenosine kinase with particular nucleoside substrate is measured by spectrophotometric assays in a coupled LDH-PK system. Assays were carried out at 50mM Tris-HCl pH7,6; 50mM KCl, 5mM MgCl2, 2,5mM ATP, 0,1mM NADH, 1mM PEP, 1mM DTT, pyruvate kinase- LDH (5U/ml each).

hAK Substrate	This work		Published		
	Km, µM	Kcat Min-1	Km, µM	Kcat Min-1	Ref
Adenosine	11	1,5	3,2	13	4
			0,150		5
Ribavirine	328	1,9	540	1,8	4
Deoxyadenosine	295	3,4	360		5
Tubercidine	12	2,2			
Inosine	1758	2,6			

Vmax and Km values of human recombinant adenosine kinase (hAK) were determined for natural substrates (adenosine, deoxy-adenosine and inosine) and nucleoside analogues. The results obtained are highly similar to previously published data.

Enzymatic characterization of human recombinant IMPDH II

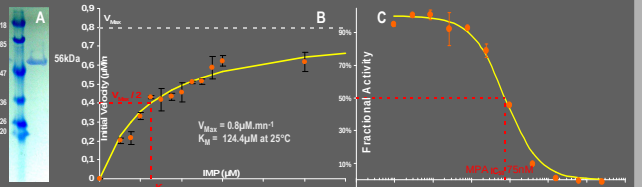

FIGURE 4: A. The cDNA encoding human IMPDH II was cloned by RT-PCR amplification of RNA from human hepatocarcinoma Huh7. The sequence of cloned IMPDH II (Accession number P22168) was confirmed by DNA sequencing (100% identity). The IMPDH II protein was overexpressed in E.coli, purified and the enzyme purity was controlled by 10% SDS-PAGE.
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.

IMPDH II enzymatic assay was validated by measuring Vmax and Km value for IMP and IC50 value for mycophenolic acid close to published data [6].

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

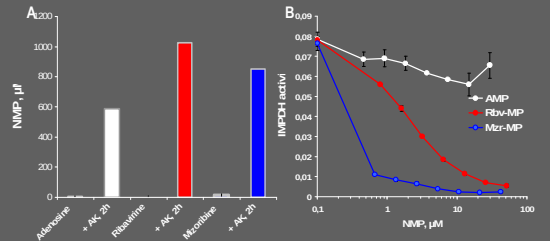

FIGURE 5: A. The initial step of nucleoside phosphorylation by hAK was followed in spectrophotometric LDH-PK system. The concentration of monophosphorylated nucleoside was quantified as ADP formed
B. The IMPDH activity was spectrophotometrically determined at 25°C by monitoring NADH formation.

Nucleoside analogues (NA) are usually designed to inhibit one viral target, the viral polymerase. Dual inhibition of viral polymerase and cellular nucleotide biosynthesis by the same NA could result in its higher efficiency due to decreased intracellular concentration of its natural competitor. For rapid evaluation of NA-monophosphate as IMPDH inhibitors, we have developed an *in vitro* enzymatic assay consisting of cloned human nucleoside kinases and cloned human IMPDH type 2. In this assay the monophosphorylation step of nucleoside analogue is provided by specific nucleoside kinases, and enzymatically produced NA-monophoshate is directly tested for IMPDH inhibition without puriifcation. This combined test has been validated with nucleoside analogues ribavirin and mizoribine, both known inhitors of human IMPDH. Depending on the nature of nucleoside analogue, this assay can be performed with other nucleoside kinases (Table 1). Morever, the effect of nucleoside analogues on nucleotide pool can be validated in whole cell bioanalytic assay (Fig. 2).

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

	Human Adenosine kinase (AK)	Human Deoxycytidine kinase (dCK)	Human cytosolic 5'-nucleotidase/ phosphotransferase II
Natural substrates	Adenosine Inosine	Deoxyadenosine Deoxyguanosine Deoxycytidine Cytidine	Deoxyinosine Inosine
Nucleoside analogues substrates	Ribavirin Tubercidin Mizoribin	Cladribine, fludarabine Gemcitabine, Lamivudine, Aracytidine Fluorodeoxyuridine	Dideoxyinosine Ribavirin Acyclovir

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