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Enzymatic assays for early characterization of novel purine nucleoside analogues

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ABSTRACT

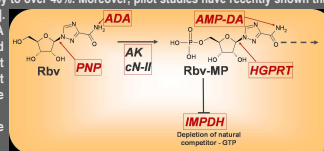
Ribavirin (RBV), purine nucleoside analogue with broad spectrum of antiviral activity, is a cornerstone of current bi-therapy of hepatitis C infection that has permitted the improvement of sustained viral response (SVR) rate in HCV patients from 10–15% for IFN- α monotherapy to over 40%. Recent clinical trials of protease inhibitor telaprevir associated with PEG-IFN- α with or without RBV have demonstrated that RBV increases SVR (62% vs 36%) and reduces the risk of selecting for resistant mutations. Despite advances in combination therapies, ribavirin modes of action still remain unclear and include: inhibition of cellular IMPDH, of viral RNA dependent polymerase, "virus mutation catastrophe" or/ and immunomodulation. The multiplicity of these modes of action probably accounts for the lack of acquired resistance to ribavirin, but it also slows down the development of new nucleoside analogues (NAs), more efficient and less toxic.

The enzymes of nucleoside metabolism may be responsible for hydrolysis of NAs and of their poor efficiency. Inhibition of these enzymes can lead to immunosuppression or toxicity. To evaluate the properties of novel NAs, we have developed a range of enzymatic assays focused on (i) human recombinant (hr) PNP, (ii) hr-HGPRT, (iii) hADA; (iv) hr-IMPDP and hr-cN-II phosphotransferases; (v) hr-IMPDP II enzyme, involved in the hydrolysis of purine NAs, their phosphorylation and / or mode of action. Since both IMPDP and HGPRT require monophosphorylated form, coupled assays (AK-IMPDP II and AK-HGPRT) were developed for rapid enzymatic synthesis of NMPs and their evaluation as IMPDP inhibitors or HGPRT substrates. The assays were validated with RBV and other nucleoside analogues.

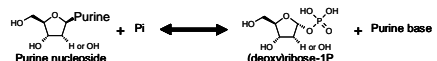
Introduction

Ribavirin is a synthetic purine nucleoside analogue with a broad spectrum of antiviral activity developed in the 70's by ICN's scientists [1]. For the last decades RBV has been mainly prescribed against severe RSV Virus. Its main current therapeutic use is, in combination with IFN- α , against HCV infection, where RBV has improved the SVR rate from 10–15% for IFN- α monotherapy to over 40%. Moreover, pilot studies have recently shown that "ribavirin priming" prior to starting combination therapy could be beneficial and could significantly increase SVR in both naive and non-responders patients [2]. Despite advances in IFN- α and RBV combination therapies, RBV modes of action still remain unclear and include: inhibition of cellular IMPDH, of viral RNA dependent polymerase, "virus mutation catastrophe", immunomodulation. The multiplicity of its modes of action may account for the lack of acquired resistance to RBV and the reduced rate of relapse after therapy cessation. This lack of viral resistance to RBV is of high importance not only for the present but also for future regimens including new specifically targeted antiviral molecules. This implies that RBV will not only remain the cornerstone of the present combination regimen but will also remain so in the new triple or quadruple therapies involving anti-HCV protease (ex. telaprevir) and anti-polymerase inhibitors which are underway [3–5].

The multiplicity of RBV modes of action (MOA) reflects our incomplete understanding of the exact mechanisms of its antiviral action. This slows down the development of new "ribavirin-like" analogues with enhanced antiviral activity and improved safety profiles.



Human Purine Nucleoside Phosphorylase



PNP (EC 2.4.2.1) is a purine salvage enzyme that catalyzes, in the presence of inorganic phosphate, the reversible reaction of hydrolysis of the glycosidic bond of ribo- or deoxyribonucleosides, to generate the purine base and ribose- or deoxyribose-1-phosphate. Because the enzyme is abundant in serum (~3mU/ml) and body organs (up to 0.45U/mg in small intestine), to be efficient, purine-based nucleoside analogues have to be resistant to PNP-catalyzed hydrolysis. From other side, PNP inhibition by nucleoside analogues may lead to T-cell specific immunosuppression or immunomodulation.

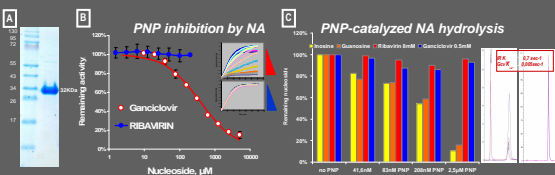


Fig 1: A. The cDNA encoding human purine nucleoside was cloned by RT-PCR amplification of mRNA extracted from human hepatoma cells and expressed in *E.coli*. The sequence of the cloned PNP (P00491) was confirmed by DNA sequencing (100% identity). The enzyme purity is controlled by 12% SDS-PAGE. B. Effect of ganciclovir (red) and ribavirin (blue) on PNP-catalyzed phosphorylation of 25µM inosine. The hydrolysis of inosine was followed spectrophotometrically at 340nm in XRR assay (NovoCIB). C. The amount of PNP-hydrolyzed nucleoside (pH 7.5, 25°C) is quantified by HPLC. The rate of NA hydrolysis (Kcat) is calculated as µM of nucleoside hydrolyzed per sec of reaction time reported to µM of enzyme concentration (40nM) and compared to that of natural substrates (inosine or guanosine).

Fig1B shows that RBV does not inhibit human PNP enzyme. Both Gcv and RBV are poor substrates for PNP. However, in contrast to ganciclovir, a known inhibitor of PNP, RBV has no effect on PNP activity.

Human Adenosine Kinase

AK (EC 2.7.1.20) catalyzes the transfer of γ -phosphate from ATP to adenosine (AR) generating AMP and ADP. AK enzyme is responsible for the phosphorylation of RBV [8], immunosuppressive drug mizoribine [9] and anticancer C-nucleoside, tiazofurin [10]. AK also controls extracellular concentration of AR, an important CNS modulator.

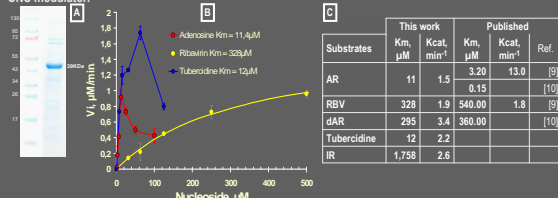


Fig 3: A. The cDNA encoding human adenosine kinase (345-aa short form, ca.39kDa) was cloned by RT-PCR amplification of mRNA extracted from human hepatoma cells and expressed in *E.coli*. The sequence of the cloned AK (U50196) was confirmed by DNA sequencing (100% identity). The enzyme purity is controlled 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-PFK 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 (50U/ml each).

Human IMP Dehydrogenase

IMPDP (E.C. 1.1.1.205) catalyzes the conversion of IMP to XMP, the pivotal step in guanine nucleotide biosynthesis. A number of NAs, such as RBV and mizoribine (MZR), inhibit IMPDP after being monophosphorylated. The therapeutic consequences of IMPDP inhibition vary for different analogues - mizoribine is an immunosuppressor and RBV is a broad spectrum antiviral. Even if direct relationship between RBV antiviral action and IMPDP inhibition by ribavirin monophosphate has not been demonstrated, the depletion of cellular GTP might result in an increased frequency of RBV-TTP incorporation by viral polymerase due to a lower intracellular concentration of its natural competitor. We have studied and compared the effect of RBV-MP, MZR-MP on IMPDP II activity in comparison with that of mycophenolic acid (MPA). We have found that RBV-MP is a ~10-fold less powerful inhibitor of IMPDP II than MPA or MZR-MP (IC₅₀ 1.6µM).

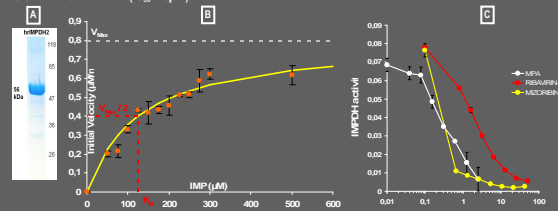
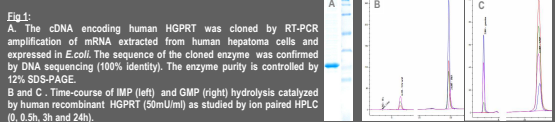


Fig 4: A. The cDNA encoding human IMPDP II was cloned by RT-PCR amplification of RNA from human hepatocarcinoma Huh7. The sequence of cloned IMPDP II (P22168) was confirmed by DNA sequencing (100% identity). The IMPDP II protein was overexpressed in *E.coli*, purified and the enzyme purity was controlled by 10% SDS-PAGE. B. Kin and Vmax values of recombinant human IMPDP II were measured spectrophotometrically by monitoring the formation of NADH at 340nm. C. Effect of RBV-MP, MZR-MP and mycophenolic acid (MPA) on hrIMPDP II (NovoCIB) RBV-MP and MZR-MP were synthesized enzymatically using hrAK (NovoCIB), and quantified by measuring ADP formed.

Human Hypoxanthine Guanine Phosphoribosyltransferase

Hypoxanthine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) is a purine salvage enzyme that catalyzes the reversible transfer of the 5-phosphoribosyl group between α -D-5-phosphoribosyl-1-pyrophosphate (PRPP) and a purine base (hypoxanthine or guanine) to form a purine nucleotide (IMP or GMP). The defects within HGPRT are associated with genetically inherited gouty arthritis and Lesch-Nyhan syndrome. In addition, HGPRT enzyme could be responsible for hydrolysis of monophosphorylated forms of NA. The study of resistance of Rbv-MP to HGPRT are currently in progress.



Human Adenosine Deaminase

ADA (ADA, EC 3.5.4.4) catalyzes the irreversible hydrolysis of adenosine to inosine (IR) and ammonia. ADA plays a dual role in nucleoside analogues pharmacology providing both inactivation and activation of nucleoside analogues.

In addition to RBV deamination [6], ADA also makes part of a strategy of pro-drugs activation, e.g. virmidine [7] or diaminopurine dioloxane [8]. Genetic defects in ADA are associated with SCID (severe combined immunodeficiency). We have determined that hADA1-catalyzed deamination of RBV is a very slow process when compared to that of adenosine (<0.001). In contrast to EHNA, RBV has no effect on adenosine hydrolysis catalyzed by hADA1 (Fig. 2).



Fig. 2: Effect of RBV and EHNA on hADA1-catalyzed hydrolysis of 50µM adenosine

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