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Note

Functional Analysis of Purine Nucleoside Phosphorylase as a Key Enzyme in Ribavirin Metabolism

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Summary: Ribavirin is a purine nucleoside analogue that possesses potent anti-hepatitis C virus activity, and it has long been considered likely that ribavirin undergoes a first-pass metabolism at the small intestine. Although purine nucleoside phosphorylase (PNP) is assumed to be involved in this metabolism, this has not been conclusively demonstrated. Furthermore, no pharmacogenomic studies related to PNP-mediated ribavirin phosphorolysis have previously been conducted. In this study, we sought to identify the role of PNP in ribavirin phosphorolysis in the human small intestine, and to clarify the effect of the single nucleotide polymorphism (rs1049564) on PNP's ribavirin phosphorolysis activity. The results of our investigations show that PNP is abundantly expressed in the human small intestine, and that intestinal ribavirin phosphorolysis is severely inhibited by ganciclovir, a PNP-inhibitor. Therefore, PNP is likely to play a primary role in the ribavirin phosphorolysis in the human small intestine. On the other hand, the results of our attempt to clarify the function of rs1049564 show that it does not affect PNP's ribavirin phosphorolysis activity. We believe that the present study will facilitate further pharmacogenomic and biochemical characterization of PNP as a key metabolic enzyme of ribavirin.

Keywords: chronic hepatitis C; phosphorolysis; purine nucleoside phosphorylase; ribavirin; single nucleotide polymorphism; small intestine; $TCONH_2$

Introduction

Ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-corboxamide) is a purine nucleoside analogue that shows potent anti-hepatitis C virus activity, and has thus been a key component of chronic hepatitis C treatment regimens.[1)] The 5′-phospate derivatives of ribavirin, which are its pharmacologically active forms, have been found to accumulate in cells at significant levels. Earlier reports have shown that higher extracellular ribavirin concentrations can be associated with higher ribavirin accumulation levels along with higher antiviral activity levels *in vitro*.[2)] Furthermore, clinical observation has shown that higher ribavirin plasma concentrations are associated with better therapeutic responses.[3)] Thus, it appears likely that the factors governing ribavirin's plasma concentrations play a pivotal role in treatment success.

Previous pharmacokinetic studies have provided results showing that the urinary excretion rate of intravenously administered ribavirin is much higher than that after oral administration, while the excretion rate of 1,2,4-triazole-3-carboxamide ($TCONH_2$), a primary metabolite of ribavirin, shows the opposite behavior, which suggests that ribavirin undergoes a first-pass metabolism.[4)] Based on the results obtained so far, the small intestine is believed to play an important role in this first-pass metabolism.[1)] Furthermore, because such first-pass metabolism levels appreciably affect the extent of bioavailability (from 28% to 85% among individuals),[5)] it is important to identify the responsible enzyme(s) along with the factors contributing to their functional variability in the small intestine. These, for the most part, remain undetermined.

The metabolism of ribavirin to $TCONH_2$ occurs in a manner similar to the endogenous purine nucleoside phosphorolysis in which purine nucleoside phosphorylase (PNP) is exclusively involved. PNP is a ubiquitously-expressed enzyme that plays a pivotal role in the purine salvage pathway.[6)] In the presence of an inorganic orthophosphate used as a second substrate, it breaks

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down the glycosidic bond of purine nucleoside (primarily inosine and guanosine in humans) to produce the purine base and ribose-(deoxyribose)-1-phosphate. Based on the nature of PNP, it was felt that a more detailed examination into the ways that human PNP impacts ribavirin's phosphorolysis activity, and thus its pharmacokinetic behavior, would be a constructive endeavor.[7] However, prior to this study, the question as to whether PNP is involved in ribavirin phosphorolysis in the human small intestine had yet to be explored. Additionally, no pharmacogenomic study of PNP regarding ribavirin metabolism had yet been performed.

In this report, we provide results that show the important role of PNP in ribavirin phosphorolysis in the human small intestine. We also show the results of a functional analysis of the single nucleotide polymorphism (SNP) in the *PNP* gene (p.G51S, rs1049564), which has demonstrated a relatively high allelic frequency, has been associated with cognitive decline in Alzheimer's disease patients, and has been associated with arsenic toxicity.[8,9]

Methods

Human samples: Pooled human jejunal S9 and pooled human liver cytosol were obtained from KAC (Kyoto, Japan) and BD Bioscience (Woburn, MA), respectively. The human jejunal cytosol was prepared from the S9 sample using a centrifugation method. The use of human samples in this study was approved by the Ethics Committee of the Graduate School of Pharmaceutical Sciences, Chiba University.

Purified PNP and PNP p.G51S preparation: PNP cDNA was cloned from human small intestine cDNA and inserted into the *Escherichia coli* (*E. coli*) expression vector pET-14b (Novagen, Madison, WI) in order to generate PNP/wt/pET. PNP cDNA harboring the SNP rs1049564 was generated by inverse-PCR, resulting in PNP/p.G51S/pET.

PNP/wt and PNP/p.G51S production in *E. coli* was separately performed essentially according to the manufacturer's protocol (Novagen). PNP/wt and PNP/p.G51S were purified from each soluble fraction using a HisTALON Gravity Columns kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. The His-tag of the purified protein was removed by thrombin treatment.

PNP activity determination: Inosine phosphorylase activity of purified PNPs or human tissue cytosols was determined using the colorimetric analysis method. The ribavirin phosphorylase activity of the purified PNPs or human tissue cytosols was examined by determining the amount of $TCONH_2$ using a high-performance liquid chromatography system. A calibration curve (0 to 10 μM) was generated using the authentic $TCONH_2$ (Tokyo Kasei, Tokyo, Japan). Ganciclovir (a known PNP inhibitor)[6] (3 mM, Sigma, St. Louis, MO), thymidine (a substrate of thymidine phosphorylase and, to a lesser extent, uridine phosphorylase) (0.5 mM, Sigma), and inosine (0.5 mM) were used in an inhibition assay. For each analysis, substrate concentrations are indicated in the figure legends.

Specific inosine or ribavirin phosphorylase activity of PNP was expressed in terms of the amount of substrate metabolized in one minute under specified conditions. Enzyme kinetic parameters were estimated using a computer program (DeltaGraph Ver 4.5, SPSS Inc., Chicago, IL), which is designed for non-linear regression analysis.

Western blotting analysis: Purified PNPs (0.1 μg each) or human tissue cytosols (40 μg each) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 5% skim milk, the membrane was incubated with either anti-PNP polyclonal antibodies (Abnova, Taipei, Taiwan) or anti-β actin antibodies (Sigma), followed by anti-mouse IgG antibodies (Sigma). The chemiluminescence was detected by LAS-1000 plus (Fuji Film, Tokyo, Japan).

Others: Method details, including other methods utilized, are provided in the supplemental materials.

Results

Ribavirin metabolic profile in the human small intestine cytosol and the human liver cytosol: To examine PNP expression in the human small intestine, Western blotting analysis was performed. The human liver cytosol was used as a comparison. The results showed that PNP expression was detected in both tissues, but the expression level in the small intestine was apparently higher than that in the liver (**Fig. 1A**). Next, the ribavirin phosphorolysis properties of these tissue cytosols were examined. Consistent with the PNP protein levels, the ribavirin phosphorolysis activity level of the small intestine was found to be significantly higher than that of the liver (**Fig. 1B**). This was also the case with the inosine phosphorylase activity level (**Supplemental Fig. s1**). Unexpectedly, we found that the reaction kinetic

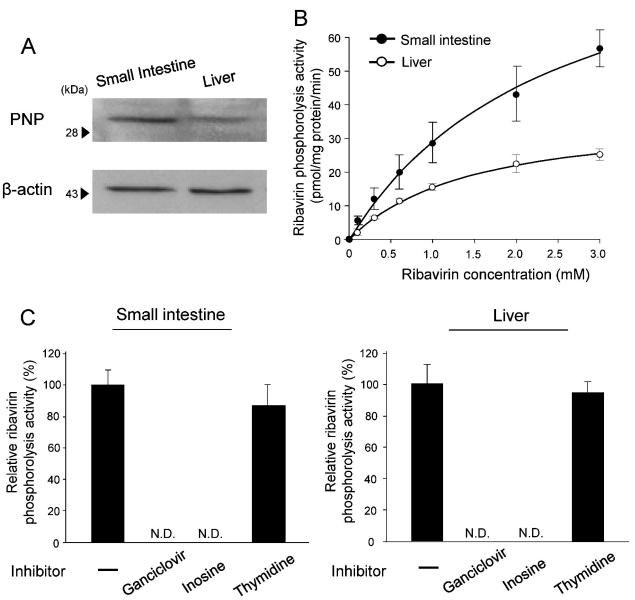

Fig. 1. Contribution of PNP to ribavirin metabolism in the human small intestine and the liver

A: PNP protein expression in the human small intestine and the liver was examined by Western blotting analysis. Each tissue cytosol (40 μg) was loaded on the gel in a separate lane. PNP protein was detected using anti-PNP antibodies, and β-actin protein expression was used as a loading control. B: Ribavirin phosphorolysis activity in the human small intestine (black circles) and the liver (white circles) was determined. Ribavirin concentrations were 0, 100, 300, 600, 1,000, 2,000, and 3,000 μM, and inorganic orthophosphate concentration was set to 50 mM. Each activity value is expressed as mean ± S.D. (pmol/mg protein/min) for three independent determinations, each performed in duplicate. C: The contribution of PNP to ribavirin phosphorolysis (2,000 μM) in the small intestine and in the liver was examined using ganciclovir (an inhibitor of PNP) (3 mM), inosine (0.5 mM) and thymidine (0.5 mM). The relative activity value in the absence of the inhibitor was set to 100%. Each value is the mean ± S.D. of relative activity for three independent experiments, each performed in duplicate. N.D., not detected.