Hepatitis C virus (HCV) is the major causative agent of non-A, non-B virus-induced hepatitis (2). An insidious and deadly disease, hepatitis C is responsible for an emerging pandemic of chronic liver diseases. There are 170 million infected individuals worldwide and approximately 4 million virus carriers in the United States alone. Unresolved acute HCV infection may progress to a chronic disease that could persist for decades. As many as 20% of infected individuals eventually develop liver cirrhosis, with 1 to 5% subsequently progressing to hepatocellular carcinoma (12). This accounts for nearly 10,000 annual deaths in the United States. The current standard for treatment is a combination therapy of subcutaneous pegylated alpha interferon with the oral nucleoside drug ribavirin (6). The sustained viral response, defined as an undetectable viral load at 6 months after cessation of therapy, is around 54 to 56% for the combination therapy. Moreover, this treatment has many adverse effects, including serious influenza-like symptoms from alpha interferon and hemolytic anemia due to the accumulation of ribavirin 5'-phosphates in red blood cells (RBCs). These undesirable side effects can lead to dose reduction and discontinuation of the combination therapy (9). In an effort to specifically deliver more ribavirin to the liver and reduce the trapping of ribavirin metabolites in RBCs, thereby improving the therapeutic index, a number of ribavirin derivatives have been explored. One promising compound that has emerged is the 3-carboxamidine derivative of ribavirin, known as viramidine. Viramidine exhibits in vitro and in vivo antiviral and immunomodulatory activities comparable to those of ribavirin (1). Recent studies revealed that viramidine mainly acts as a prodrug and is converted to ribavirin by adenosine deaminase (Fig. 1) (14). Animal studies indicate that viramidine is not efficiently taken up by RBCs like ribavirin (5). In contrast, viramidine has a better liver-targeting property and is enriched in the liver twice as much as ribavirin (13). Owing to this favorable property of enrichment in the liver, as well as a reduced exposure to the risk of hemolysis development, viramidine appears to be a safer alternative to ribavirin, which could potentially provide improved clinical benefits to HCV patients. Viramidine is currently in phase 3 clinical trials with pegylated alpha interferon for the treatment of active chronic HCV infection.

Purine nucleoside phosphorylase has been reported to metabolize ribavirin to triazole nucleobase in vivo as illustrated in Fig. 1 (7). Conversely, viramidine is not a substrate but an inhibitor for nucleoside phosphorylase (11). Therefore, we reason that viramidine could potentially prevent ribavirin from catabolism by inhibiting nucleoside phosphorylase. To investigate this novel concept, a purine nucleoside phosphorylase from human blood was obtained from Sigma. A radiochemical-based thin-layer chromatography (TLC) assay was developed to monitor the conversion of [5-14C]ribavirin (54 mCi/mmol; Moravek Biochemicals, Brea, Calif.) to [5-14C]triazole nucleobase. In the assay, nucleoside phosphorylase (2.5 U/ml) was added to 10 µl of 1× Dulbecco’s phosphate-buffered saline, pH 7.4, containing various concentration of ribavirin. The assay mixture was incubated for 10 min at 30°C and then was stopped by heating at 90°C for 1 min. The assay mixture was briefly clarified by centrifugation. Four microliters of the reaction mixture was applied to a silica gel 60 TLC plate (Selecto Scientific, Suwanee, Ga.), which was then developed in a solvent system of chloroform-methanol-acetic acid (85:15:5). The TLC plate was dried and autoradiographed overnight. Products on the TLC plate were analyzed and quantified with a PhosphorImager. With this assay, we found that nucleoside phosphorylase indeed catalyzes phosphorylation of ribavirin as previously reported (7). However, under similar conditions, [5-14C]viramidine (56 mCi/mmol; Moravek Biochemicals) was not hydrolyzed, indicating that viramidine is not a substrate for purine nucleoside phosphorylase.

Further steady-state kinetic analysis showed that the reaction of ribavirin phosphorylase was linear for the first 15 min and it quickly reached equilibrium within half an hour (data not shown). At equilibrium, approximately 40% of the ribavirin was converted, confirming that the phosphorylase process is reversible and nucleoside phosphorylase catalyzes the reaction in both directions. The initial velocity at various concentrations of ribavirin (0.2 to 2 mM plus 0.054 µCi of [5-14C]riba-
virin) was determined and applied to the Michaelis-Menten equation with a nonlinear least-squares fit to calculate the $k_{cat}$ and $K_m$ values for the reaction. The $K_m$ for ribavirin was determined to be $0.76 \pm 0.08$ mM from an average of three results (Fig. 2A). To calculate $k_{cat}$, the human blood purine nucleoside phosphorylase from Sigma was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and its purity was estimated at around 60%. With the assumption that this commercial nucleoside phosphorylase is fully active, the $k_{cat}$ was then calculated as $33 \pm 3$ min\(^{-1}\). Thus, ribavirin is a decent substrate for purine nucleoside phosphorylase, with a catalytic efficiency of $43$ min\(^{-1}\)mM\(^{-1}\).

Inhibition of ribavirin phosphorolysis by viramidine was studied by varying the inhibitor’s concentration from 1 to 25 $\mu$M with the ribavirin concentration fixed at $100$ $\mu$M. Applying the initial velocities at different inhibitor concentrations to a Dixon plot yielded a $K_i$ of $2.5 \pm 0.1$ $\mu$M for viramidine (Fig. 2B). This is similar to the reported $K_i$ for viramidine when viramidine was tested against human lymphoblast purine nucleoside phosphorylase with inosine as a substrate (11). In addition, we investigated viramidine 5'-monophosphate (VMP), a major metabolite of viramidine, as an inhibitor for nucleoside phosphorylase. Inhibition of VMP against human blood purine nucleoside phosphorylase was performed by titrating VMP from 10 to 1,250 $\mu$M against a fixed concentration of ribavirin (100 $\mu$M). From a Dixon plot, the $K_i$ for VMP was calculated to be around $250$ $\mu$M. This result indicates that VMP inhibits purine nucleoside phosphorylase about 100-fold weaker than does viramidine. The weak inhibitory activity of VMP may not be physiologically relevant. From these studies, we conclude that viramidine is a potent inhibitor for purine nucleoside phosphorylase and it is capable of preventing ribavirin phosphorolysis in vitro.

Previous drug action mechanism studies suggest that viramidine confers the majority of its antiviral activity through the prodrug mechanism. Its immunomodulatory activity observed in peripheral blood mononuclear cells or in vivo animal models is likely derived from ribavirin that is generated from deamination of viramidine (10). This study further reveals a potential self-potentiating catabolic inhibition mechanism of viramidine. Our in vitro data convincingly demonstrated that viramidine inhibits ribavirin phosphorolysis with good potency. This in vitro study has significant in vivo implications, considering the oral delivery route of viramidine through the stomach and intestines and drug transportation from the plasma to the liver, in some of which nucleoside phosphorylase is highly expressed.

![FIG. 1. Schematic diagram depicting viramidine as a prodrug and as a catabolic inhibitor for ribavirin. Ribavirin is subject to either 5' phosphorylation by nucleoside and nucleotide kinases or degradation to nucleobase by purine nucleoside phosphorylase. In addition to functioning as a prodrug of ribavirin, viramidine could directly inhibit nucleoside phosphorylase and prevent or slow down the catabolism of the newly converted ribavirin, thereby providing more ribavirin for phosphorylation.](http://aac.asm.org/)
may warrant further clinical considerations with a combination therapy of ribavirin and a nucleoside phosphorylase inhibitor, such as viramidine, to achieve higher potency and efficacy in the treatment of chronic HCV infection. This study also provides a new concept in the design of bifunctional prodrugs.

We acknowledge J. Shim, C.-C. Lin, W. Zhong, D. Smith, R. Tam, and H. Walker for helpful discussion and suggestions.

REFERENCES