Intestinal Absorption of Ribavirin Is Preferentially Mediated by the Na^+-Nucleoside Purine (N1) Transporter

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INTRODUCTION

Nucleoside transport across cell membranes is mediated by multiple transporters (1). Recently, we reported the presence of two Na^+-dependent concentrative nucleoside transporters (N1 and N2) on the brush border and two Na^+-independent equilibrative nucleoside transporters (*es* and *ei*) on the basolateral membrane of the human intestine (2,3). The N1 (*cif*) transporter is generally purine specific; guanosine, formycin B and inosine serve as model substrates. The N2 (*cit*) transporter is pyrimidine specific; thymidine serves as a model substrate. Uridine and adenosine are transported by both the N1 and N2 systems (4). Both the equilibrative nucleoside transporters (*es* and *ei*) have broad substrate specificity, but differ in their sensitivity to inhibition by nitro-benzyl-mercaptopurine riboside (NBMPR) (5,6).

Ribavirin (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a broad-spectrum antiviral agent structurally related to guanosine, has activity in vitro and in vivo against both RNA and DNA viruses (7) and respiratory syncytial virus (RSV) (8,9). Following administration of an oral solution or a capsule, the mean bioavailability of ribavirin is ~40% (10–12). Pharmacokinetic studies of oral ribavirin in adult subjects have demonstrated a lack of proportionality in the peak plasma concentrations achieved on increasing the dose from 600 to 2400 mg, suggesting saturable absorption of the drug (13). Since ribavirin is a structural analogue of guanosine, we hypothesized that it is a substrate for the N1 (*cif*) transporter which we have previously shown to be expressed in the human intestine (2). If this hypothesis is correct, saturable absorption of ribavirin could be explained by saturation of its transport by the N1 Na^+-nucleoside transporter. To test this hypothesis, we conducted studies on the uptake of ribavirin by the brush border membrane vesicles (BBMV) obtained from the human intestine.

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MATERIALS AND METHODS

Chemicals

^{3}H-thymidine (20 Ci/mmol) and ^{3}H-inosine (40 Ci/mmol) were obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA); ^{3}H-ribavirin (10 Ci/mmol) and unlabeled ribavirin were kindly provided by the Schering-Plough Research Institute (Kenilworth, NJ). All other chemicals were of the highest analytical grade available.

Transport Studies

BBMV were prepared from 3 to 4 human jejunum as described earlier (2). Pooled membrane vesicles were resuspended in 50 mM HEPES-Tris buffer (pH 7.4) containing 0.1 mM $MgSO_4$, 200 mM KCl and 3 μM valinomycin. The purity of the BBMV was routinely monitored by measuring alkaline phosphatase and Na^+-K^+-adenosinetriphosphatase (ATPase) activities in both the starting homogenate and BBMV. Influx of ^{3}H-labeled substrates was initiated by the addition of 10 μl of BBMV to 40 μl of incubation medium containing final concentrations of 150 mM NaCl, 50 mM KCl, 0.1 mM $MgSO_4$ and 50 mM HEPES-Tris buffer (pH 7.4) at room temperature. Passive uptake of the nucleoside into the BBMV was measured by substituting 150 mM of KCl for NaCl in the above uptake assay. Uptake assay was terminated at 10 s by rapid-filtration inhibitor-stop method as described earlier (2). All experiments were conducted in duplicate or triplicate. The IC_{50} and Michaelis-Menten data were analyzed using nonlinear regression (WinNONLIN).

RESULTS AND DISCUSSION

Protein content and marker enzyme activities were measured in both the mucosal homogenates and the BBMV. The activity of the brush-border marker enzyme, alkaline phosphatase, was enriched 15-fold and the basolateral marker enzyme, Na^+-K^+-ATPase, was diminished about 50% with respect to the starting homogenate (data not shown).

In the presence of a Na^+-gradient (150 mM, out > in), ^{3}H-ribavirin (1 μM) uptake showed a transient overshoot which tapered to an equilibrium value at approximately 15 minutes (Fig. 1). In the absence of a Na^+-gradient, the overshoot phenomenon was abolished. There was no significant difference between equilibrium uptake values in the presence or absence of the Na^+-gradient, suggesting that there was little or no difference in the integrity and membrane permeability of vesicles under both conditions. These data indicate that ribavirin uptake is mediated by a carrier protein which is driven by a Na^+ gradient.

To determine the relative contributions of the N1 and N2 transporters to ribavirin uptake by the jejunal BBMV, we measured the effect of selective inhibitors of the N1 (inosine) or N2 (thymidine) transporters on the uptake of ribavirin. ^{3}H-ribavirin (2 μM) uptake by the jejunal BBMV was almost completely inhibited (93 ± 8%) by inosine (100 μM) but was not affected (94 ± 8% of control value remaining) by thymidine (100 μM) (Fig. 2). In the same batch of vesicles and at the concentration used (100 μM), both inosine and thymidine were confirmed to be selective inhibitors of the N1 and the N2

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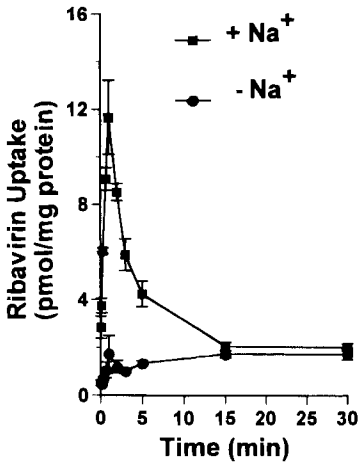


Fig. 1. The time course of ^{3}H-ribavirin (1 μM) uptake by brush-border membrane vesicles (BBMV) isolated from the human jejunum in the presence (■) or absence (●) of a Na^+-gradient (150 mM, out > in). Points shown are the means ± SD of triplicate determinations.

transporters, respectively (Fig. 2). That is, inosine significantly inhibited the uptake of tracer inosine without affecting the uptake of tracer thymidine. Similarly, thymidine significantly inhibited the uptake of tracer thymidine without affecting the uptake of tracer inosine. These findings suggest that the uptake of ribavirin (at least at tracer concentrations) is primarily mediated by the N1 nucleoside transporter.

To further characterize these interactions, we determined the inhibitory potencies (IC_{50} values) of ribavirin towards the uptake of inosine and thymidine and that of inosine and thymidine towards the uptake of ribavirin. Ribavirin was ~15-fold more potent in inhibiting inosine uptake than in inhibiting thymidine uptake (IC_{50}: 16.8 and 16.3 μM vs. 241 ± 106 μM; n = 3) by the jejunal BBMV (Fig. 3A). Conversely, inosine was ~400-fold more potent than thymidine in inhibiting ribavirin uptake (IC_{50}: 3.2 and 3.1 μM vs. 1327 ± 230 μM; n = 3) by

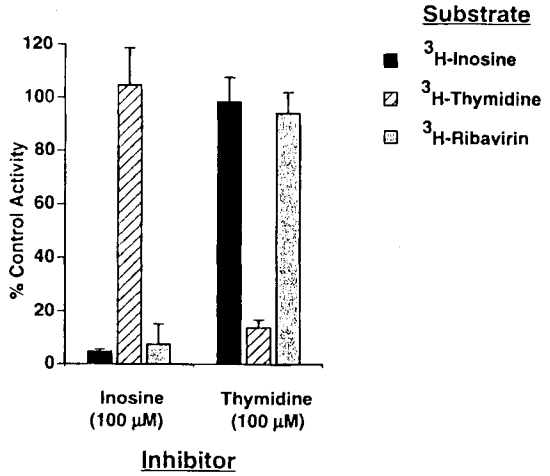
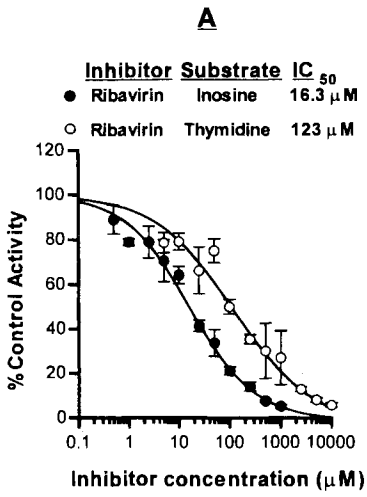


Fig. 2. Uptake of ^{3}H-ribavirin (2 μM), ^{3}H-inosine (0.5 μM), and ^{3}H-thymidine (1μM), in the presence of selective inhibitors of the N1 (inosine) or N2 (thymidine) Na^+-nucleoside transporters in pooled human jejunal BBMV. Each bar represents mean ± SD of net Na^+-dependent uptake after correcting for diffusion.



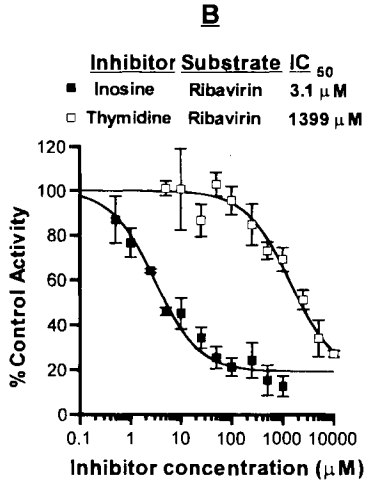


Fig. 3. The inhibitory potencies (IC_{50}) of ribavirin on the uptake of the N1 and N2 substrates, inosine (0.5 μM) and thymidine (1 μM), by the jejunal BBMV were determined (panel A). Also determined were the inhibitory potencies of inosine and thymidine towards the uptake of ribavirin (2 μM) by the jejunal BBMV (panel B). Data are means ±SD of triplicate determinations. Profiles shown were obtained from the same batch of pooled vesicles and are representative of experiments carried out on 2 to 3 separate batches of pooled vesicles. The IC_{50} values listed are those of the profiles shown.

the jejunal BBMV (Fig. 3B). These observations further support the conclusion that ribavirin uptake is preferentially mediated by the N1 Na^+-dependent transporter in human jejunal BBMV. The similarity in the inhibitory potency of inosine towards ribavirin uptake and ribavirin towards inosine uptake suggests that both substrates are taken up by the N1 transporter with a similar affinity. Indeed this is the case. Ribavirin was transported by the N1 transporter with an affinity (K_m: 5.0 and 11.2 μM; V_{max}: 25.9 and 28.4 pmol/mg protein/10s) comparable to the affinity with which the N1 transporter transports inosine (K_m: 5.3 and 5.3 μM; V_{max}: 133 and 119 pmol/mg protein/10s) and guanosine (K_m: 12.0 ± 1.3 μM; V_{max}: 64 ± 11 pmol/mg protein/10s; n = 3) (2). Moreover, by Lineweaver-Burk analysis followed by nonlinear regression, ribavirin was found to be a competitive inhibitor of inosine uptake with a K_i value of 19.4

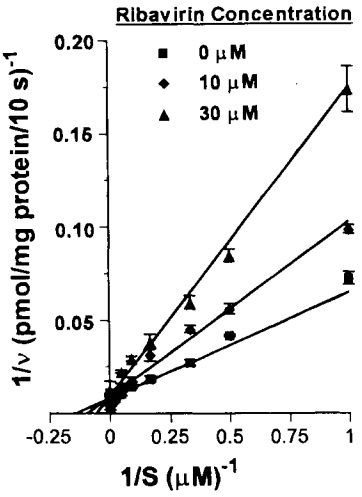


Fig. 4. Lineweaver-Burk plot of the Na^+-dependent uptake of inosine in the human intestinal BBMV in the presence of several concentrations of ribavirin. Data shown are corrected for Na^+-independent inosine uptake. The K_i value, as estimated by nonlinear regression, was 19.4 ± 5.0 μM.

μM (Fig. 4). As would be expected for a competitive inhibitor, this K_i value is similar to the IC_{50} value. Surprisingly, ribavirin was found to be a more potent inhibitor of thymidine uptake than was thymidine of ribavirin uptake. This discrepancy suggests that ribavirin is either a substrate of the N2 transporter or it is a noncompetitive or uncompetitive inhibitor of this transporter. The data presented in Figure 2 indicate that the uptake of tracer ribavirin is abolished by inosine at a concentration (100 μM) at which inosine does not affect the uptake of an N2 substrate (thymidine). That is, uptake of ribavirin at tracer concentrations appears not to be mediated by the N2 transporter. However, at higher concentrations of ribavirin (>100 μM), the N1 transporter will approach saturation and the N2 transporter, if it transports ribavirin, may begin to contribute to the uptake of ribavirin. In this case, the affinity of the N2 transporter for ribavirin will approximate the IC_{50} value (~241 μM). Alternatively, as indicated above, ribavirin may be a noncompetitive or an uncompetitive inhibitor of this transporter. These hypotheses will be best differentiated by conducting ribavirin uptake studies with the cloned N2 transporter.

We have shown that ribavirin is transported across the brush border membrane of the human jejunum by the N1 Na^+-nucleoside transporter. Assuming that a dose of ribavirin (600 mg) is completely and instantaneously dissolved in the human small intestine [volume of fluid = 1.65 L (14)], the concentration of ribavirin in the intestinal lumen will be ~1500 μM. At this high concentration, which is much greater than the K_m value of ribavirin transport by the N1 Na^+-nucleoside transporter (and also possibly of the N2 Na^+-nucleoside transporter), the absorption of the drug by the N1 transporter (and potentially by the N2 transporter) should be saturable. Hence, we propose that the saturable uptake of ribavirin in the human intestine is the most likely explanation for the observation that the maximal plasma concentration of ribavirin after oral administration of the drug (600–2400 mg) does not increase in proportion with the dose (13).

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