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Ribavirin uptake by human erythrocytes and the involvement of nitrobenzylthioinosine-sensitive (*es*)-nucleoside transporters

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1 The major toxicity associated with oral therapy with ribavirin is anaemia, which has been postulated to occur as a result of accumulation of ribavirin triphosphate interfering with erythrocyte respiration. The objective of this study was to determine the mechanism by which ribavirin enters into erythrocytes.

2 Entry into human erythrocytes was examined by measuring influx rates of [^{3}H]-ribavirin alone and with the inhibitor nitrobenzylthioinosine (NBMPR), and by investigating the inhibitory effects of nucleoside and nucleobase permeants on ribavirin transport, by use of inhibitor oil-stop methods. Transport mechanisms were further characterized by assessment of substrates to cause countertransport of ribavirin in preloaded erythrocytes, and by measuring the effects of ribavirin on [^{3}H]-NBMPR binding to erythrocyte membranes.

3 Human erythrocytes had a saturable influx mechanism for ribavirin (K_m at 22°C of 440 ± 100 μM) which was inhibited by nanomolar concentrations of NBMPR (IC_{50} 0.99 ± 0.15 nM). Nucleosides also inhibited the influx of ribavirin (adenosine more effective than uridine) but the nucleobases hypoxanthine and adenine had no effect. In addition, uridine caused the countertransport of ribavirin in human erythrocytes. Entry of ribavirin into horse erythrocytes, a cell type that lacks the NBMPR-sensitive (*es*) nucleoside transporter, proceeded slowly and via a pathway that was resistant to NBMPR inhibition. Ribavirin was a competitive inhibitor of adenosine influx (mean K_i 0.48 ± 0.14 mM) and also inhibited NBMPR binding to erythrocyte membranes (mean K_i 2.2 ± 0.39 mM).

4 These data indicate that ribavirin is a transported permeant for the *es* nucleoside transporter of human erythrocytes. There was no evidence for ribavirin entering cells via a nucleobase transporter.

Keywords: Ribavirin; adenosine; nitrobenzylthioinosine; nucleoside transporter; erythrocyte

Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-trizole-3-carboxamide) is a broad-spectrum antiviral agent that is active both *in vivo* and *in vitro* against a variety of viral infections including hepatitis C, influenza, respiratory syncytial virus and Lassa fever (DiBisceglie *et al*., 1992; Sidwell *et al*., 1972; Hall *et al*., 1985; McCormick *et al*., 1986; Knight *et al*., 1981). At present, a number of clinical studies are underway to confirm preliminary findings that combined ribavirin plus interferon α-2b is more effective against hepatitis C virus than interferon α-2b alone (Brillanti *et al*., 1994; Lai *et al*., 1996; Reichard *et al*., 1996). The main toxicity associated with ribavirin administration is a dose-related anaemia that is reversible upon cessation of the treatment (DiBisceglie *et al*., 1992). Pharmacokinetic studies have shown that ribavirin can be taken up by human erythrocytes (Laskin *et al*., 1987) and reaches steady state concentrations that exceed the plasma concentration by up to 60 fold (Lertora *et al*., 1991). The high concentration of ribavirin inside red cells is due to ribavirin being phosphorylated to ribavirin triphosphate which accumulates as dephosphorylation in erythrocytes is ineffective (Catlin *et al*., 1980; Page & Connor, 1990; Letora *et al*., 1991). The exact mechanism for ribavirin inducing anaemia is unknown, but it may be due to the extensive phosphorylation of ribavirin interfering with cellular-ATP mediated energy processes and reducing cell viability (Shulman, 1984).

The above studies clearly indicate that ribavirin, a structural analogue of guanosine, enters human erythrocytes, but there is no information on the route of entry. Extensive studies with many nucleoside analogues have shown that they are substrates for the nitrobenzylthioinosine (NBMPR)-sensitive (*es*) nucleoside transporter in human erythrocytes (Cass, 1995; Griffith & Jarvis, 1996). However, some guanosine analogues, notably acyclovir and 2′3′-dideoxyguanosine, are permeants for the facilitated-diffusion nucleobase transporter of human erythrocytes (Mahony *et al*., 1988; Gati *et al*., 1992). The aim of this study was to determine the mechanism(s) by which ribavirin is transported into human erythrocytes.

Methods

Erythrocytes

Blood from healthy human volunteers and horses was collected into heparin-treated tubes. Recently outdated blood (stored as red cells at 4°C in citrate/phosphate/dextrose supplemented with an additive solution containing 150 mM NaCl, 1.25 mM adenine, 46.4 mM glucose and 28.6 mM mannitol) was obtained from the National Blood Service, U.K. Blood was centrifuged at 1500 *g* for 10 min and the plasma and buffy coats were discarded. The erythrocytes were washed three times with 20 vol. of medium containing 140 mM NaCl, 5 mM KCl, 20 mM Tris-HCl (pH 7.4 at 22°C), 2 mM $MgCl_2$ and 0.1 mM EDTA (disodium salt). Red cell counts and haematocrit estimations were performed by established methods (Archer, 1965). Haemoglobin free 'ghosts' were prepared by standard procedures as described previously (Jarvis & Young, 1980).

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Nucleoside transport

The influx of nucleosides by human erythrocytes at 22°C was measured by mixing 0.15 ml portions of cell suspension (haematocrit 20%) with 0.05 ml of medium containing the appropriate concentration of radioactive nucleoside. In inhibition studies, with the exception of NBMPR, test compound and radioactive nucleoside were added simultaneously. In the case of NBMPR, 0.1 ml of cells were incubated with 10 ml of varying concentrations of NBMPR. After 30 min, the cells were centrifuged, the supernatant removed and 0.2 ml of radioactive medium supplemented with the appropriate concentration of NBMPR added. At specified time intervals, transport was terminated by the inhibitor-oil stop method (Jarvis *et al*., 1980; 1982). Portions of the incubation medium (0.15 ml) were added to 0.75 ml of ice-cold stop medium containing 20 μM dilazep, layered on top of 0.5 ml ice-cold di-*n*-butyl phthalate contained in 1.5 ml microcentrifuge tubes. Tubes were immediately centrifuged (13,000 *g*, 10 s), and the aqueous and di-*n*-butylphthalate layers removed by aspiration and the inside of the tube wiped dry, leaving a cell pellet at the bottom of the tube. Radioactivity associated with the cell pellet was determined with a Beckman or Wallac scintillation counter with automatic quench correction and disintegrations min^{-1} conversion (Young, 1978). Blank values (radioactivity that became associated with cells during an uptake interval of zero time) were obtained by processing cell samples exposed simultaneously to radioactively labelled nucleoside and 10 μM NBMPR at 1°C. Transport rates were calculated after subtraction of the blank. The intracellular water space of the erythrocytes was determined from the difference in the total water space and the extracellular water space by use of ^3H_2O and [U-^{14}C]-sucrose, respectively in place of radioactive nucleoside in the uptake assay, and centrifuging the cells directly through the di-*n*-butyl phthalate in the absence of stop solution.

In other experiments, the ability of test compounds to cause the inward countertransport of radioactive labelled ribavirin was investigated. Washed erythrocytes were first 'loaded' with test permeant by incubating cells (0.1 ml) with 1.0 ml of test compound for 30 min. After 30 min at 22°C, cells were pelleted (10 s, 13,000 *g*) in a microcentrifuge and the supernatant removed. To remove the remaining traces of extracellular test permeant, the cells were then rapidly washed with 1.0 ml of ice-cold medium, and without delay, 0.2 ml of [^{3}H]-ribavirin was added to the cell pellet with concurrent vortex mixing. At predetermined time intervals, 0.2 ml samples of the incubation mixture were removed and added to the stop tubes and processed as described above.

In experiments with horse erythrocytes which exhibit slow uptake fluxes of nucleosides (Jarvis & Harris, 1997), experiments were conducted at 37°C and the transport assays were terminated after 5 min by adding 1 ml portions of ice-cold medium containing 10 μM NBMPR. The cells were pelleted and then rapidly washed four times with 1 ml ice-cold portions of stop medium, by use of a microcentrifuge (10 s, 13,000 *g*). Previous control experiments had established that this washing procedure removed extracellular labelled permeant without significant loss of radioactivity from cells with slow uptake rates (Young, 1978).

Nitrobenzylthioinosine binding

Human erythrocyte ghosts (0.2 mg of protein), prepared from outdated blood, were incubated at 22°C in 5 mM sodium phosphate buffer, pH 7.9, in the presence of 1 nM [^{3}H]-NBMPR and varying concentrations of test compound (total volume 2.0 ml). Incubations (30 min) were terminated by filtration over Whatman GF/B filters presoaked for at least 1 h in 0.3% (v/v) polyethylenimine and washed twice with 4 ml aliquots of ice-cold sodium phosphate buffer. Specific binding is defined as the difference in membrane content of radioactivity in the presence and absence of 10 μM NBMPR.

Data analysis

All experiments were carried out in triplicate unless stated otherwise and the errors in the Figures are standard errors. In least square fits to the data, points were weighted according to the inverse of their relative s.e. Inhibitor and kinetic constants (IC_{50} and K_m) were calculated from curves by use of the Fig P computer program (Elsevir Biosoft). K_i values were calculated from the equation $K_i = IC_{50}/(1 + (L/K_m \text{ or } K_d))$, in which L is the ligand concentration for either the transport or NBMPR binding studies.

Chemicals

[2,8,5′-^{3}H]-adenosine (65 Ci $mmol^{-1}$) and ^3H_2O (25 mCi g^{-1}) were purchased from NEN. [U-^{14}C]-sucrose (23.2 mCi $mmol^{-1}$) and [G-^{3}H]-NBMPR (36 Ci $mmol^{-1}$) were obtained from Amersham and Moravek Biochemicals, respectively. [G-^{3}H]-ribavirin (9 Ci $mmol^{-1}$) and ribavirin were generous gifts from Schering-Plough Research Institute, New Jeresy. All other reagents were from Sigma.

Results

Inhibition of [^{3}H]-adenosine influx by ribavirin

In the first series of experiments the ability of ribavirin to interact with the nucleoside transporter was determined by indirect methods. Figure 1 shows the effect of ribavirin on adenosine influx at 22°C by human erythrocytes from outdated blood. The results demonstrate that ribavirin was a relatively low affinity competitive inhibitor of adenosine influx with an apparent K_i value of 470 μM. In other experiments, dose-response curves with ribavirin for inhibition of adenosine influx (5 μM) were monophasic (Hill coefficient of -0.89 ± 0.07) with an IC_{50} value of 483 ± 142 μM (mean $\pm$ s.e.mean, $n = 3$). Self-inhibition of adenosine influx by adenosine, conducted with the same blood samples, revealed an IC_{50} value of 48 ± 5 μM with a Hill coefficient of -0.94 ± 0.09 (mean $\pm$ s.e.mean, $n = 3$).

[^{3}H]-NBMPR binding

NBMPR is a potent and specific inhibitor of nucleoside transport, inhibition resulting from the binding of NBMPR to high-affinity sites on the nucleoside transporter (Jarvis & Young, 1980; Cass, 1995; Griffith & Jarvis, 1996). Ribavirin inhibited site-specific steady state binding of 1 nM [^{3}H]-NBMPR to unsealed erythrocyte membranes in a dose-dependent manner with an IC_{50} value of 4.5 ± 0.8 mM and a Hill coefficient of -1.07 ± 0.17 (average of three independent estimates). Using a previously determined estimate of K_d (0.97 nM) (Jarvis *et al*., 1983a), the K_i for ribavirin inhibition of NBMPR binding was determined to be 2.2 ± 0.39 mM. In the same experiments, adenosine displaced [^{3}H]-NBMPR binding with an apparent K_i value of 370 ± 59 μM.

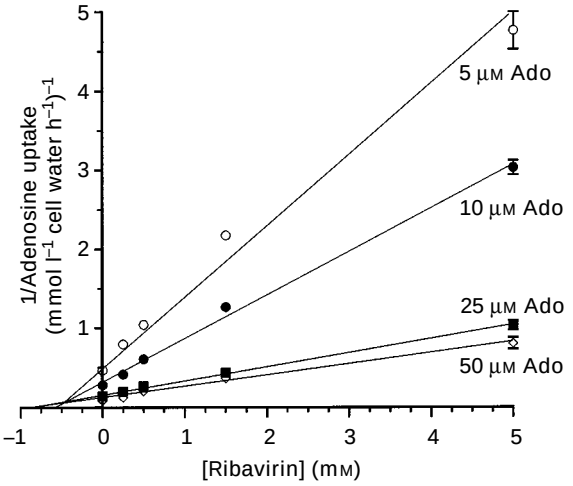

Figure 1 Dixon plot of ribavirin inhibition of adenosine (Ado) influx by human erythrocytes. The reciprocals of initial rates of adenosine influx (5–50 μM) at 22°C are plotted against the respective concentrations of inhibitor (ribavirin). Apparent K_i was 0.47 mM.

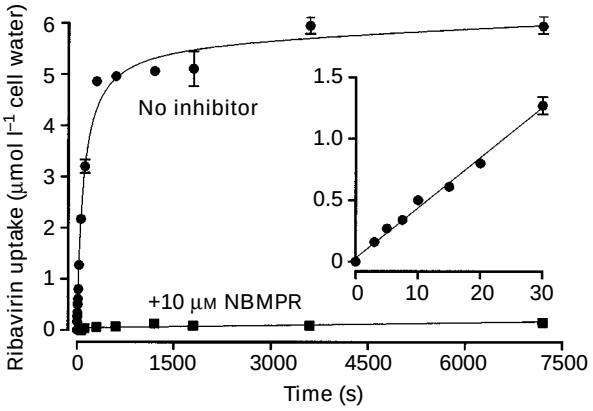

Figure 2 Time course of ribavirin uptake by human erythrocytes from outdated blood at 22°C. Intervals of 5 μM ribavirin influx were initiated as described in Methods in the presence or absence of 10 μM NBMPR, and terminated by the inhibitor-oil-stop method. Inset shows the uptake of ribavirin over the first 30 s with the axis titles and units the same as on the main graph.

Uptake of [^{3}H]-ribavirin

The above data are consistent with the idea that ribavirin interacts with the permeant site of the *es* nucleoside transporter. However, these results provide no conclusive information on whether ribavirin is a transported permeant for the *es* carrier. Both transported and non-transported substrates could be expected to inhibit adenosine transport and NBMPR binding in a competitive manner. To distinguish between a transported and a non-transported permeant, the direct transport of isotopically labelled ribavirin was measured. These studies are also important to determine if there are multiple routes of entry of ribavirin influx in human erythrocytes.

Zero-*trans* influx of 5 μM ribavirin by erythrocytes from fresh blood at 22°C was rapid and within 1 min the intracellular concentration was equal to that of the extracellular concentration. NBMPR at 10 μM inhibited the initial rate of influx by greater than 99% (1910 and 4.5 μmol l^{-1} cell water h^{-1}, respectively), whereas 10 mM ribavirin reduced the initial rate of influx by 97% (43 μmol l^{-1} cell water h^{-1}). Similar results were obtained with 100 μM [^{3}H]-ribavirin (data not shown). In subsequent kinetic studies with fresh human erythrocytes, initial ribavirin uptake rates were determined by use of a 3 s incubation period.

An earlier study (Jarvis *et al*., 1983a) had demonstrated that in erythrocytes from outdated blood, the K_m value for zero-*trans* uridine influx was similar to that observed in fresh erythrocytes, but the V_{max} was reduced by four fold. The influx of ribavirin was similarly reduced in erythrocytes from outdated blood compared with fresh erythrocytes. Figure 2 demonstrates that influx of 5 μM [^{3}H]-ribavirin was linear for at least 30 s with a rate of 152 μmol l^{-1} cell water h^{-1}, a value 8% of that seen with fresh erythrocytes. Nevertheless, NBMPR inhibited ribavirin influx by greater than 99% (0.6 μmol l^{-1} cell water h^{-1}). Initial rates of [^{3}H]-ribavirin influx by erythrocytes from outdated blood were determined with a 10–20 s incubation period. Due to the availability of virus-free outdated blood, and the relatively long incubation times ($\sim$15 s) that will result in estimates of initial rates of ribavirin influx with human erythrocytes from outdated blood, subsequent studies were mainly conducted with outdated blood.

The data of Figure 2 suggest that ribavirin enters human erythrocytes via a mediated route that is blocked by NBMPR. To confirm that there is only a single mediated route with characteristics expected of the *es* nucleoside transporter, the potency of NBMPR to inhibit [^{3}H]-ribavirin influx was examined. Figure 3 shows that NBMPR inhibits [^{3}H]-ribavirin influx by erythrocytes from outdated blood in a monophasic manner with an IC_{50} value of 0.99 nM, a potency similar to that observed for NBMPR inhibition of adenosine and uridine transport in human erythrocytes (Jarvis *et al*., 1982; 1983b; Paterson *et al*., 1984). The mean IC_{50} value from 3 experiments was 0.99 ± 0.15 nM (Hill coefficient -1.27 ± 0.24) and was similar to a single value obtained for erythrocytes from fresh blood (4.1 nM). The concentration-dependence of NBMPR-sensitive ribavirin influx by human erythrocytes from outdated blood is shown in Figure 4. Uptake of ribavirin was saturable and conformed to simple Michaelis-Menten kinetics, giving an apparent K_m value of 420 ± 67 μM with a V_{max} estimate of 30 ± 1.6 mmol l^{-1} cell water h^{-1}. The mean kinetic constants for ribavirin influx from 3 different blood samples were apparent K_m 440 ± 100 μM with a V_{max} of 38 ± 12 mmol l^{-1} cell water h^{-1}. This apparent K_m value was almost identical to the apparent K_i value for ribavirin inhibition of adenosine influx suggesting adenosine and ribavirin are transported by the same system. A similar K_m value was obtained with fresh erythrocytes (630 ± 140 μM), but the V_{max} was increased by $\sim$7 fold (290 ± 16 mmol l^{-1} cell water h^{-1}).

To investigate further the substrate specificity of ribavirin influx into human erythrocytes, the ability of nucleosides and nucleobases to inhibit the influx of ribavirin was examined. Adenosine was shown to be a more effective inhibitor than uridine with apparent K_i values of 57 ± 5 and 134 ± 20 μM (mean $\pm$ s.e.mean ($n = 3$)), respectively. These K_i values are similar to previous estimates of the apparent K_m values for adenosine and uridine influx by human erythrocytes (Jarvis *et al*., 1983a; Paterson *et al*., 1984; Plagemann *et al*., 1985) supporting the suggestion that ribavirin, adenosine and uridine are transported by the same system in human erythrocytes. In contrast, hypoxanthine had no effect on 5 μM ribavirin influx by fresh erythrocytes (2.11 ± 0.08 and 2.07 ± 0.02 mmol l^{-1} cell water h^{-1} in the presence and absence of 0.5 mM hypoxanthine). An alternative means to test the specificity of the

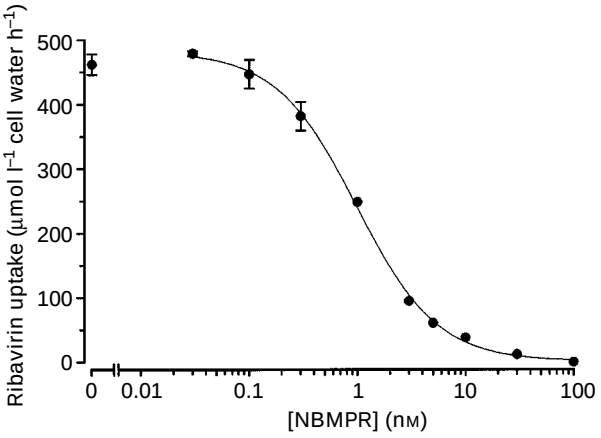

Figure 3 Effects of NBMPR on ribavirin uptake by human erythrocytes. The initial rates of 5 μM ribavirin influx in the presence of NBMPR were determined as described in Methods. Values are the means and vertical lines s.e.mean of triplicate estimates from a single experiment. The IC_{50} value $= 0.99 \pm 0.05$ nM and Hill coefficient $= -1.16 \pm 0.05$.

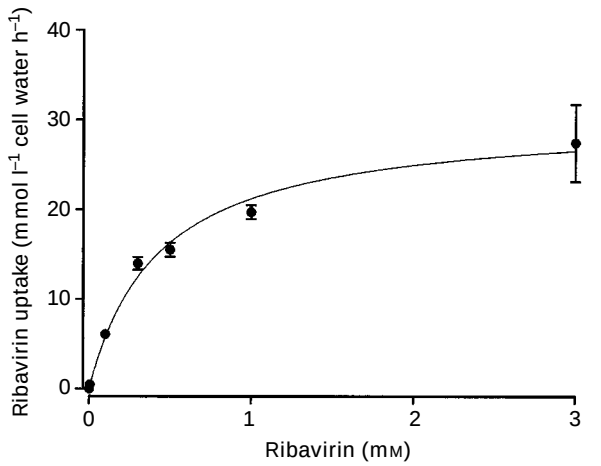

Figure 4 Concentration-dependence of mediated ribavirin uptake by human erythrocytes. Human erythrocytes at 22°C were incubated with [^{3}H]-ribavirin and increasing amounts of unlabelled ribavirin and initial rates of ribavirin influx determined. Non-mediated ribavirin influx was estimated from the residual flux in the presence of 20 mM ribavirin and subtracted from the total ribavirin uptake values. Non-linear regression analysis of the data shown gave kinetic constants of 420 ± 67 μM for the K_m with a V_{max} of 30 ± 1.6 mmol l^{-1} cell water h^{-1}.

ribavirin uptake pathway is to test the ability of compounds to effect the countertransport of ribavirin (Figure 5). Preincubation of human erythrocytes with 1 mM uridine or ribavirin resulted in a transient accumulation of [^{3}H]-ribavirin in which the maximum intracellular concentration of [^{3}H]-ribavirin (9 μmol l^{-1} cell water) was approximately 1.8 fold higher than the extracellular concentration. In contrast, the time courses of [^{3}H]-ribavirin uptake by cells incubated previously with adenine were indistinguishable from those of cells with exposure to buffer alone.

Previous studies (Jarvis & Harris, 1998) have demonstrated that horse erythrocytes do not possess a saturable nucleoside transport system. In the present investigation, horse erythrocytes transported 5 μM ribavirin with a slow rate (1.2 μmol l^{-1} cells h^{-1}) which was not inhibited by 5 μM NBMPR (influx rate 1.4 μmol l^{-1} cells h^{-1}).

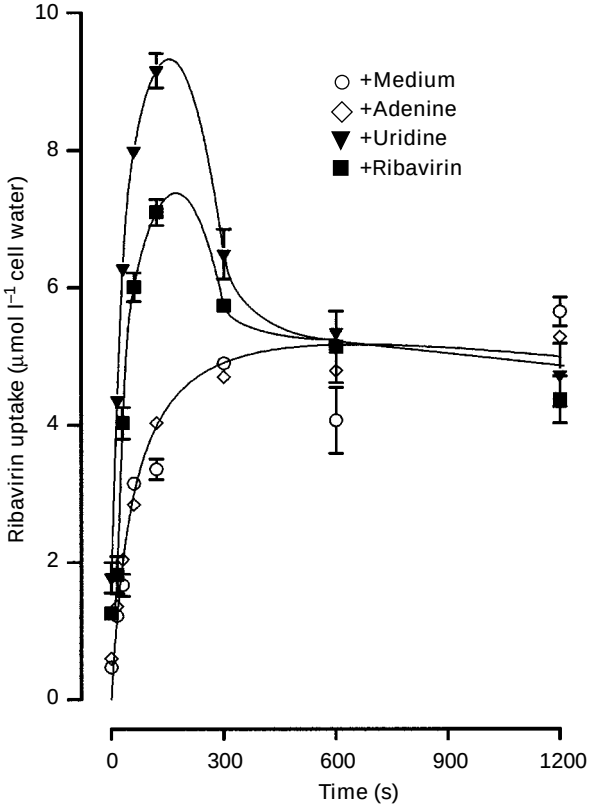

Figure 5 Countertransport of ribavirin after preloading human erythrocytes with uridine or ribavirin. Human erythrocytes were preloaded with 1 mM uridine, or 1 mM ribavirin, or 1 mM adenine, or incubation medium; washed free of extracellular test compound; and then resuspended in [^{3}H]-ribavirin (final concentration 5 μM). Cell-associated [^{3}H]-ribavirin was measured as a function of time.

Discussion

The present study used two complementary approaches to determine the mode of entry of ribavirin in erythrocytes. In the first, the interaction of non-radioactively labelled ribavirin with the nucleoside carrier present in human erythrocytes was characterized. The second approach was to measure directly the transport of radioactively labelled ribavirin by mammalian erythrocytes.

Human erythrocytes were shown to possess a saturable influx mechanism for ribavirin (K_m at 22°C of approximately 440 μM). Uptake by this route was inhibited by nanomolar concentrations of NBMPR with an IC_{50} value of 0.99 ± 0.15 nM. Nucleosides also inhibited the influx of ribavirin but hypoxanthine and adenine had no effect. Adenosine was a more effective inhibitor than uridine, a result that is consistent with the known relative affinities of these two nucleosides for the human erythrocyte nucleoside transporter (Griffith & Jarvis, 1996). In addition, uridine caused the countertransport of ribavirin in human erythrocytes. All of these results suggest that the transport of ribavirin by human erythrocytes is mediated by the *es* nucleoside transporter. Further support for this conclusion comes from the finding that entry of ribavirin into horse erythrocytes, a cell type that lacks the *es* carrier, proceeds slowly and via a pathway that is resistant to NBMPR inhibition. In addition, ribavirin was demonstrated to be a competitive inhibitor of adenosine influx, and also an inhibitor of NBMPR binding to erythrocyte membranes. The apparent K_i value for ribavirin inhibition of

NBMPR binding to human erythrocyte membranes was 2.2 ± 0.39 mM, a value higher than the apparent K_i value for inhibition of adenosine influx (0.48 ± 0.14 mM). A similar apparent discrepancy was noticed for adenosine inhibition of NBMPR binding and ribavirin influx (370 ± 59 versus 57 ± 5 μM). Previous experiments have also noted that the apparent K_i value for nucleoside inhibition of site-specific NBMPR binding is higher than the K_m for influx, but close to the K_m for equilibrium exchange (Jarvis *et al*., 1982; 1983b).

Although the majority of the present studies were conducted with human erythrocytes obtained from outdated blood, similar results were obtained with the limited studies performed on fresh erythrocytes. In particular, the affinity of ribavirin for zero-*trans* influx was similar for erythrocytes from fresh and outdated blood (K_m values of 630 ± 140 μM compared to 440 ± 100 μM). However, the velocity of ribavirin influx by fresh erythrocytes was 5 to 10 fold greater than that for erythrocytes from outdated blood. These characteristics, no change in K_m but a lower rate of influx in erythrocytes from outdated blood compared to fresh erythrocytes for ribavirin, are identical to earlier findings with uridine (Jarvis *et al*., 1983a).

Multiple transporters for nucleosides have been recognized and characterized on the basis of substrate specificity, inhibitor sensitivity, ion requirements, and more recently gene sequence (Cass, 1995; Griffith & Jarvis, 1996; Griffiths *et al*., 1997). Thus, although all the data presented here are consistent with the notion that ribavirin is a transported permeant for the *es* nucleoside transporter of human erythrocytes, the applicability of this finding to other cell types and other nucleoside transporters remains to be determined. Nevertheless, unlike other guanosine analogues that have been suggested to be permeants for the erythrocyte nucleobase carrier (Mahony *et al*., 1988; Gati *et al*., 1992), hypoxanthine failed to inhibit ribavirin influx and adenine did not cause the countertransport of [^{3}H]-ribavirin. Thus, there is no evidence for ribavirin entering cells via a nucleobase transporter.

Earlier studies had shown that prolonged treatment of patients with ribavirin resulted in the accumulation of ribavirin in the form of phosphorylated metabolites in their erythrocytes (Catlin *et al*., 1980; Laskin *et al*., 1987; Page & Connor, 1990; Lertora *et al*., 1991). Our data demonstrate that over short periods of time (up to 120 min), the intracellular concentration of ribavirin did not exceed the extracellular concentration, suggesting that the phosphorylation of ribavirin is a slow process in human erythrocytes from outdated blood. Thus, it may be theoretically possible to prevent the accumulation of ribavirin in human erythrocytes, and thus reduce or prevent the associated anaemia, by inhibiting ribavirin uptake with the use of *es* nucleoside transporters such as NBMPR, dipyridamole or dilazep. This strategy of coadministration of transport inhibitor and nucleoside analogue has been demonstrated to be successful for certain chemotherapeutic regimes (Griffith & Jarvis, 1996). Nevertheless, if the inhibition of ribavirin uptake is less than 100% then ribavirin will still enter cells and over extended periods of treatment, e.g. days, would be expected to accumulate in erythrocytes. Moreover, as *es* nucleoside transporters are widely distributed (Cass, 1995; Griffith & Jarvis, 1996), the addition of nucleoside transport inhibitor may also prevent entry of ribavirin in those cells infected with hepatitis C virus thereby decreasing the efficacy of ribavirin treatment.

In conclusion, the data presented here demonstrate that ribavirin is a transported permeant for the *es* nucleoside transporter of human erythrocytes.

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