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Transport of ribavirin in human myelogenous leukemia cell line K562

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The anticancer effect of ribavirin, a purine nucleoside analogue, has been studied using cultured cancer cells such as the human myelogenous leukemia cell line K562. In order to exert its pharmacological effect, ribavirin has to enter cancer cells. However, there is little information concerning the transport mechanism of ribavirin into K562 cells. In this study, therefore, we examined the uptake mechanism of ribavirin in K562 cells. The uptake of ribavirin in K562 cells was time- and temperature-dependent, and was saturable with a Km value of 1.5 mM. Ribavirin uptake was inhibited by nucleosides such as adenosine and uridine, and by inhibitors of equilibrative nucleoside transporter 1 (ENT1) such as S-(4-nitrobenzyl)-6-thioinosine and dipyridamole in a concentration-dependent manner. In addition, the expression of ENT1 mRNA in K562 cells was confirmed by real-time PCR. On the other hand, Na^+-dependence of ribavirin uptake was not observed, suggesting the involvement of ENT1, but not Na^+-dependent concentrative nucleoside transporters, in ribavirin uptake in K562 cells. Treatment of K562 cells with sodium butyrate induced erythroid differentiation, but ribavirin uptake activity and sensitivity of the uptake to various inhibitors were not different between native and differentiated K562 cells. These results suggest that ribavirin uptake into K562 cells is mainly mediated by ENT1, which may have a pivotal role in anticancer effect of ribavirin.

1. Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a purine nucleoside analogue which is used for the treatment of various viral infections such as hepatitis C virus and respiratory syncytial virus infections (Dusheiko et al. 1996; Hijano et al. 2019 ; Thong et al. 2015). Especially, for the treatment of chronic hepatitis C, a combination of ribavirin with interferon α-2b or peginterferon α-2b has long been a standard therapy. The major side effect of ribavirin is hemolytic anemia, and the plasma concentration of ribavirin during pharmacotherapy is closely associated with its antiviral effect as well as its side effects including anemia (De Franceschi et al. 2000). We have previously reported that the high steady-state trough plasma concentration of ribavirin of approximately 4000 ng/mL decreased hemoglobin concentrations to less than 8.5 g/dL in Japanese chronic hepatitis C patients, and suggested that dosage adjustment of ribavirin based on renal function and body weight would provide safer and more effective treatment without causing hemolysis (Maeda et al. 2004).

Though ribavirin has been used as an antiviral agent in clinical pharmacotherapy for a long time, more recent studies have demonstrated anticancer properties of ribavirin. Anticancer properties of ribavirin have been studied in *in-vitro* and *in-vivo* models and in clinical trials in multiple cancers including acute myeloid leukemia (Borden and Culjkovic-Kraljacic 2010; Casaos et al. 2019; Urtishak et al. 2019). Ribavirin may inhibit an oncogene eIF4E, the eukaryotic translation initiation factor, which is upregulated in approximately 30% of cancers including many leukemias and lymphomas, and thereby suppress cancer cell proliferation (Borden and Culjkovic-Kraljacic 2010; Casaos et al. 2019). Another target of ribavirin is inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the guanosine biosynthetic pathway (Borden and Culjkovic-Kraljacic 2010; Franchetti and Grifantini 1999; Kökény et al. 2009). Type II IMPDH is upregulated in neoplastic and replicating cells, and inhibition of IMPDH by nucleoside inhibitors such as ribavirin and tiazofurin, another nucleoside analogue, would result in depletion of the guanylate pools and exert anticancer effect (Franchetti and Grifantini 1999). K562 cells are often used to study the anticancer effect of ribavirin (Kökény et al. 2009; Shi et al. 2015) as well as tiazofurin and its analogs (Kojić et al. 2019; Weber et al. 1990). K562 is a cell line derived from human chronic myelogenous leukemia with positive Philadelphia chromosome (Lozzio and Lozzio 1975). Kökény et al. (2009) reported that treatment of K562 cells with ribavirin resulted in a significant growth inhibition *via* activating apoptosis and the differentiation pathway, and concluded that ribavirin is a potent anticancer agent. In a more recent study (Shi et al. 2015), ribavirin was shown to have anti-proliferation effect on K562 cells mediated by downregulation of phosphorylation levels of various key molecules including eIF4E protein in the mTOR/eIF4E signaling pathway. In addition, the combination of ribavirin with imatinib enhanced above-mentioned anticancer effects of ribavirin on K562 cells. Similar effects of ribavirin were observed in Philadelphia chromosome positive acute lymphoblastic primary leukemic blasts obtained from acute leukemia or chronic myelogenous leukemia patients. Thus, ribavirin alone or in combination with other anticancer drugs may be a promising strategy for leukemia therapy.

In order to exert its anticancer effect via interaction with various intracellular molecules, the entry of ribavirin into cancer cells should be prerequisite. Ribavirin is a hydrophilic molecule, and therefore cannot permeate the lipid bilayer of cell membranes by a passive diffusion. Therefore, in this study, we examined the uptake mechanisms of ribavirin in K562 cells. In addition, erythroid differentiation of K562 cells is known to be induced by several compounds such as sodium butyrate (NaBu) and IMPDH inhibitors (Li et al. 2008; Yu et al. 1989). It should be interesting to compare ribavirin transport in native and differentiated K562 cells. Therefore, the effect of erythroid differentiation on ribavirin transport was examined using native and differentiated K562 cells.

2. Investigations and results

2.1. Time-, temperature- and concentration-dependence of ribavirin uptake in K562 cells

First, we examined time- and temperature-dependence of ribavirin uptake in K562 cells. At 23 °C, ribavirin uptake increased with time, but the uptake was very rapid and reached an equilibrium after about 30 s. The uptake at ice-cold temperature (ICT; 0.5–0.7 °C) also showed time-dependence, but the uptake rate was significantly lower than that at 23 °C (Fig. 1A).

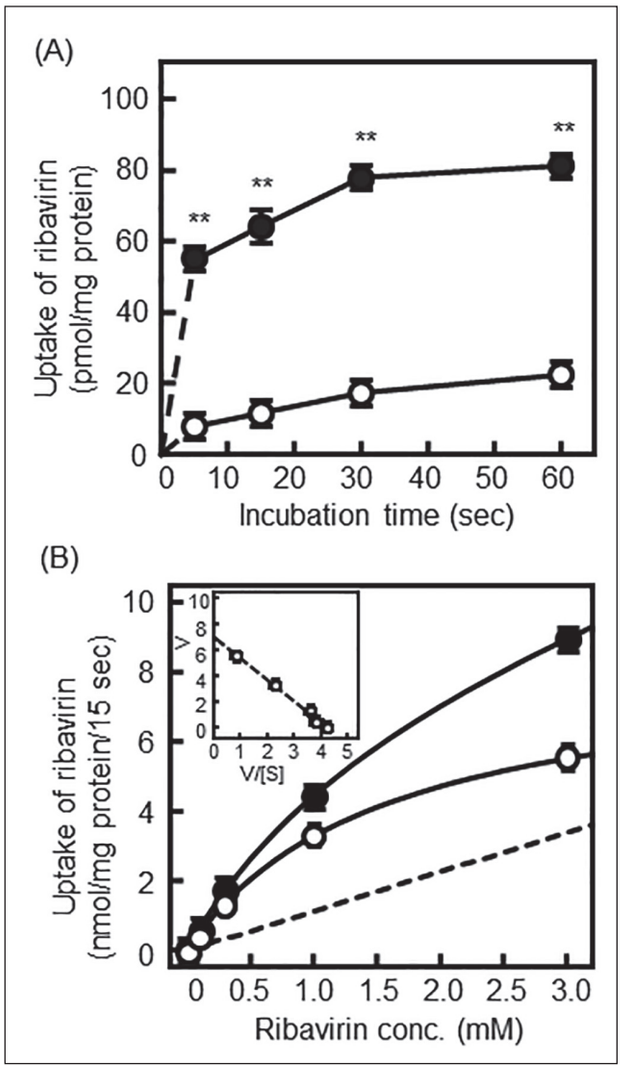

Fig. 1: Time-, temperature-, and concentration-dependence of [^{3}H]ribavirin uptake in K562 cells. (A) K562 cells were incubated with 15 μM [^{3}H]ribavirin at 23 ºC (●) or ice-cold temperature (ICT, ○). $^{**}p < 0.01$, significantly different from each value at ICT. (B) K562 cells were incubated with various concentrations of ribavirin (0.01–3.0 mM) for 15 s at 23 ºC. Solid lines indicate the fitting curves for total uptake (●) and saturable uptake (○). The dashed line indicates the non-saturable component. The inset shows Eadie-Hofstee plots of saturable uptake of ribavirin. Each point represents the mean ± S.E.M. (n=4).

Figure 1B shows the concentration-dependence of ribavirin uptake in K562 cells. The uptake of ribavirin for 15 s was measured at various concentrations, and was found to be saturable at high ribavirin concentrations. Ribavirin uptake in K562 cells was suggested to be mediated by a single transport system, as evidenced by the linear Eadie-Hofstee plot (Fig. 1B, inset). K_m (Michaelis-Menten constant), V_{max} (maximum uptake rate), and K_d (diffusion constant) values were calculated to be 1.5 mM, 8.4 nmol/mg protein/15 s, and 1.1 μL/ mg protein/15 s, respectively, using a Michaelis-Menten type equation.

2.2. Effects of substrates and inhibitors of ENT on ribavirin uptake in K562 cells

The effect of nucleosides, adenosine and uridine, on ribavirin uptake was examined (Fig. 2A, B). Both nucleosides inhibited ribavirin uptake in a concentration-dependent manner. The IC_{50} values of adenosine and uridine on ribavirin uptake were 107.4 μM and 201.3 μM, respectively.

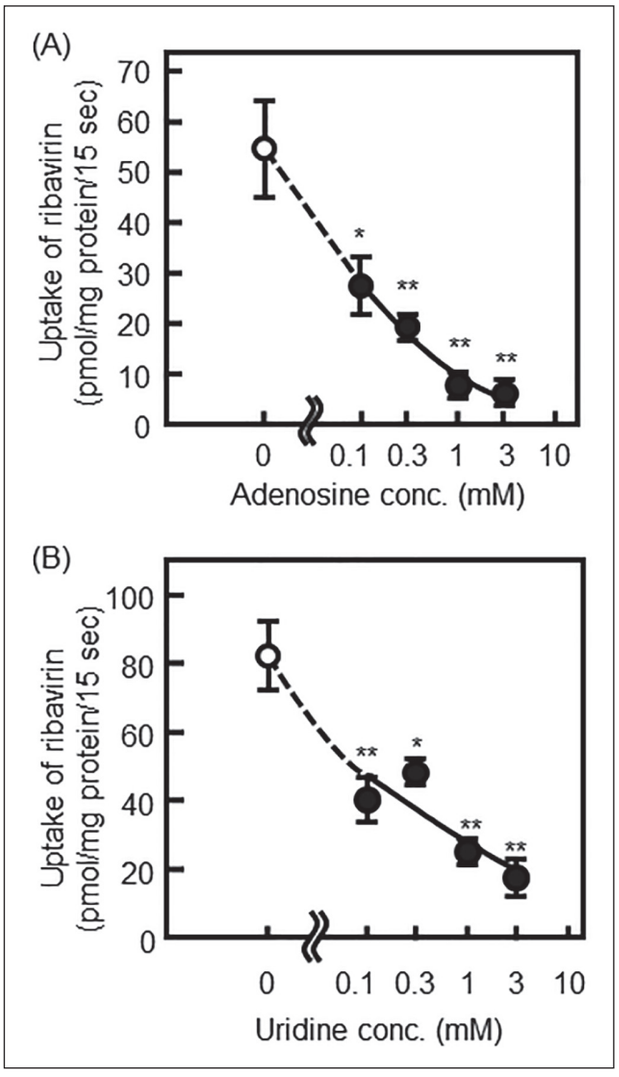

Fig. 2: Effect of adenosine and uridine on [^{3}H]ribavirin uptake in K562 cells. K562 cells were incubated with 15 μM [^{3}H]ribavirin in the absence (control, ○) or presence (●) of various concentrations of (A) adenosine or (B) uridine. Solid lines indicate the fitting curves calculated using the Hill equation. Each point represents the mean±S.E.M. (n=4). $^*p< 0.05$, $^{**}p < 0.01$, significantly different from each control.

We further examined the effect of S-(4-nitrobenzyl)-6-thioinosine (NBMPR) and dipyridamole, inhibitors of ENT1 (Fig. 3A, B). These compounds inhibited ribavirin uptake in a concentration-dependent manner. The IC_{50} values of NBMPR and dipyridamole on ribavirin uptake were 397.7 nM and 432.9 nM, respectively. In the experiment the cells were co-incubated with ribavirin and inhibitors during the uptake period (15 s). When K562 cells were preincubated with NBMPR for 10 min and then uptake experiment was performed, the inhibitory effect of NBMPR on ribavirin uptake became more potent (about 60% inhibition by 10 nM (NBMPR, data not shown). NBMPR is known to selectively inhibit ENT1 at nM concentrations, suggesting that ribavirin uptake in K562 cells is mainly mediated by ENT1.

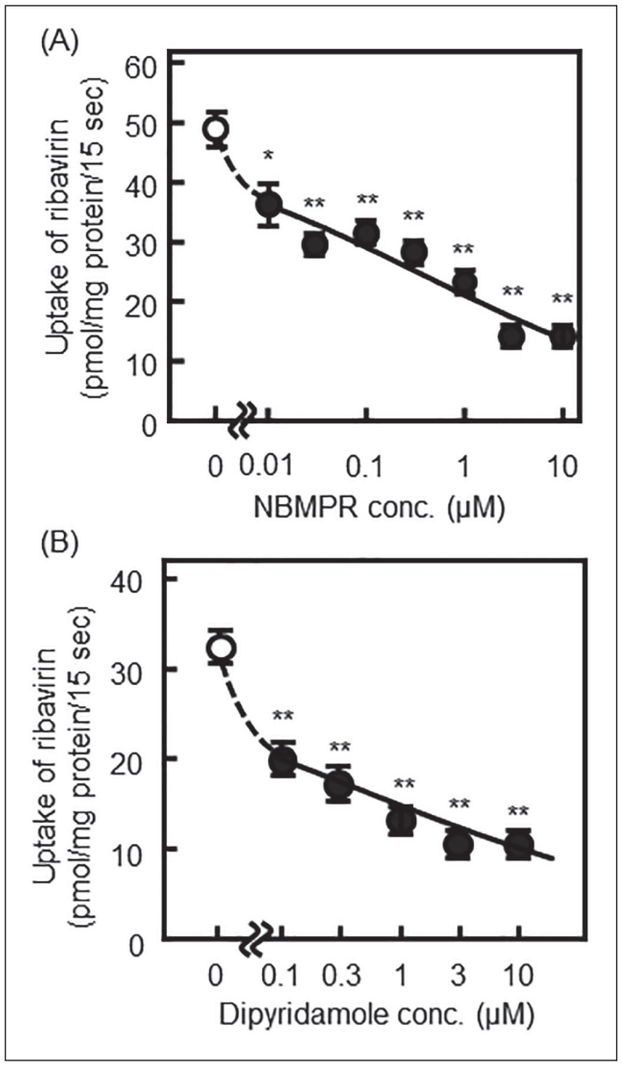

Fig. 3. Effect of NBMPR and dipyridamole on [^{3}H]ribavirin uptake in K562 cells. K562 cells were incubated with 15 μM [^{3}H]ribavirin in the absence (control, ○) or presence (●) of various concentrations of (A) NBMPR or (B) dipyridamole. Solid lines indicate the fitting curves calculated using the Hill equation. Each point represents the mean ± S.E.M. (n=4). *p< 0.05, **p < 0.01, significantly different from each control.

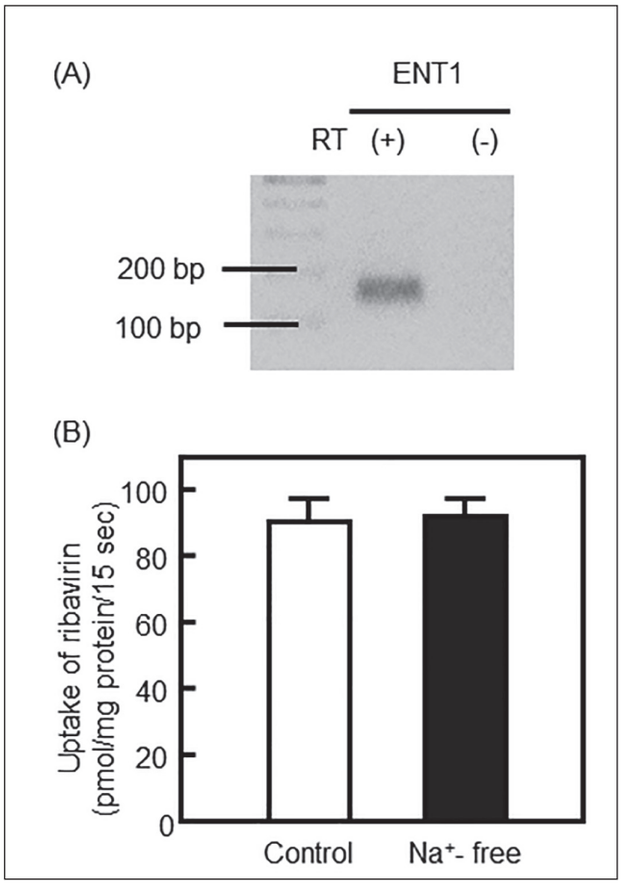

Fig. 4: The mRNA expression of ENT1 and sodium dependence of [^{3}H]ribavirin uptake in K562 cells. (A) Total mRNA was extracted from K562 cells and the mRNA expression of ENT1 was determined using real-time PCR. PCR products with (+) or without (-) reverse transcription (RT) were separated by electrophoresis. (B) K562 cells were incubated with 15 μM [^{3}H]ribavirin in the presence of sodium chloride (control, open column) or choline chloride (closed column). Each value represents the mean±S.E.M. (n=4).

2.3. Expression of ENT1 mRNA and Na$^+$-dependence of ribavirin uptake in K562 cells

In order to confirm ENT1 expression in K562 cells, the expression of ENT1 mRNA was examined by real-time PCR. As shown in Fig. 4A, ENT1 mRNA expression was clearly detected in K562 cells. Figure 4B shows the effect of extracellular Na^+ on ribavirin uptake. The uptake rate of ribavirin did not change in the presence or absence of Na^+, indicating that Na^+-dependent concentrative nucleoside transporters (CNTs) would not be involved in ribavirin uptake in K562 cells.

2.4. Erythroid differentiation of K562 cells

NaBu treatment is known to induce erythroid differentiation in K562 cells. In order to compare ribavirin uptake in native and differentiated K562 cells, we first examined whether differentiation can be induced by NaBu under our experimental conditions. As shown in Fig. 5A, the color of the cells became red by NaBu treatment, most likely due to the synthesis of hemoglobin. In addition, NaBu treatment markedly increased the mRNA expression of glycophorin A, another marker of erythroid differentiation (Fig. 5B). Thus, erythroid differentiation of K562 cells induced by NaBu treatment was confirmed.

2.5. Effect of erythroid differentiation on ribavirin uptake in K562 cells

Using native and differentiated K562 cells, the uptake of ribavirin was compared at low and high concentrations of ribavirin (15 μM and 1.5 mM). However, ribavirin uptake activity at both concentrations was not different between these K562 cells (Fig. 6). In addition, ribavirin uptake in differentiated K562 cells was inhibited by adenosine, uridine, NBMPR, and dipyridamole (Fig. 7), as observed in native K562 cells. These results indicate that erythroid differentiation of K562 cells would not affect ribavirin transport mediated by ENT1.

3. Discussion

Though K562 leukemia cells have been used to study the anticancer effect of ribavirin, no information concerning the transport mechanism of ribavirin in K562 cells was available. In this study, we found that ribavirin was taken up by a specific transport system, most likely by ENT1, in K562 cells. Using human choriocarcinoma BeWo cells, Yamamoto et al. (2007) studied the transport mechanism of ribavirin, and suggested that ribavirin was taken up by both Na^+-dependent transporter CNT3 and Na^+-independent ENT1 and ENT2. More recently, Karbanova et al. (2019) examined ribavirin uptake in BeWo cells as well as in fresh human placental villous fragments and microvillous plasma membrane vesicles. They observed the involvement of CNTs in ribavirin uptake in BeWo cells, but not in placental villous fragments and microvillous plasma membrane vesicles, and concluded that riba-

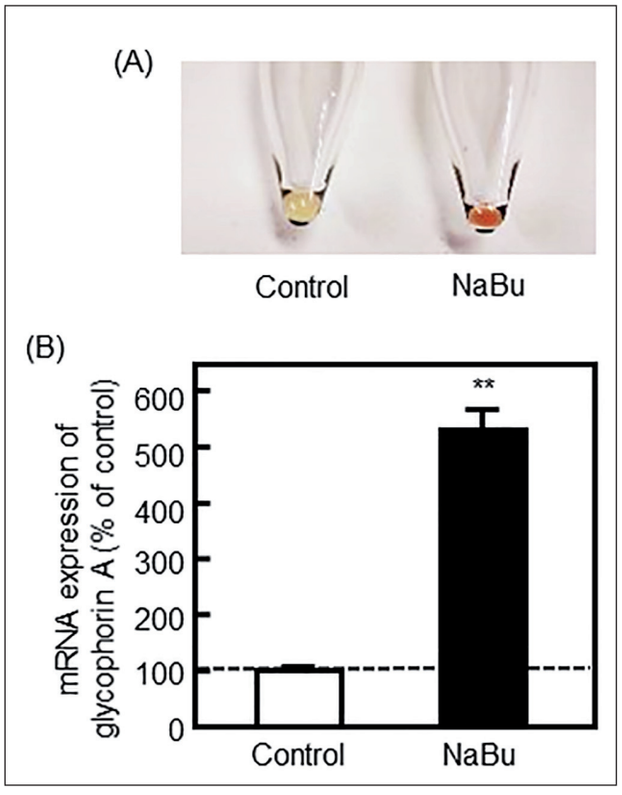

Fig. 5: Effect of NaBu treatment on the color of cells and mRNA expression level of glycophorin A in K562 cells. K562 cells were cultured without (control) or with 1 mM NaBu for 4 days. (A) Cells were centrifuged and photographed. (B) mRNA expression level of glycophorin A was analyzed by real-time PCR, which was normalized by GAPDH mRNA expression. Each value represents the mean±S.E.M. (n=3). $^{**}p < 0.01$, significantly different from control.

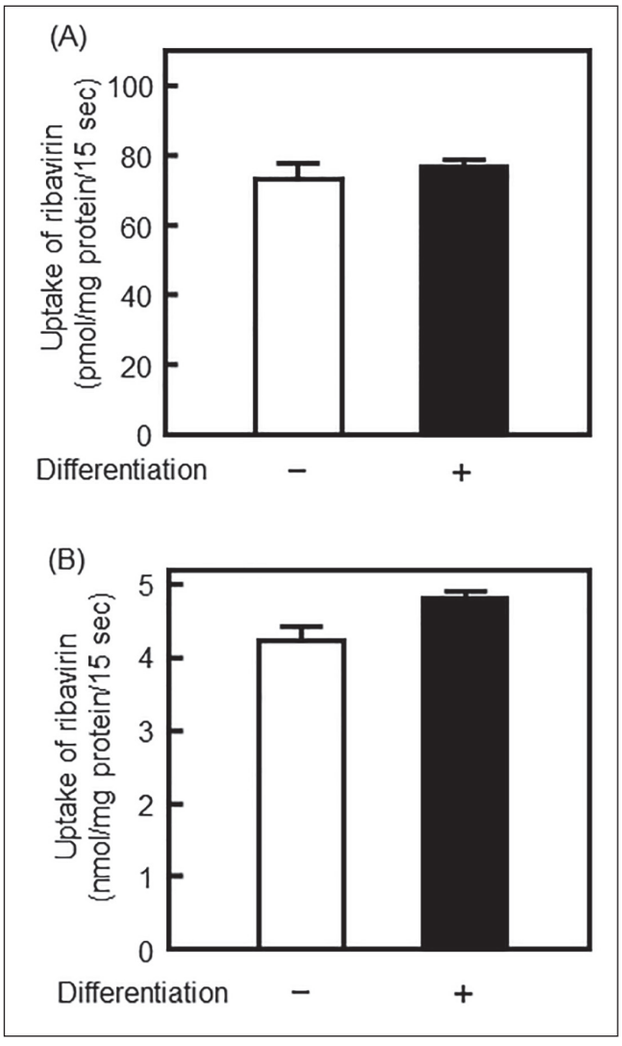

Fig. 6: Effect of erythroid differentiation on [^{3}H]ribavirin uptake in K562 cells. K562 cells were cultured without (–, open column) or with 1 mM NaBu (+, closed column) for 4 days. Cells were incubated with [^{3}H]ribavirin at a concentration of (A)15 μM and (B) 1.5 mM. Each value represents the mean±S.E.M. (n=4).

virin transport in placental barrier was mainly mediated by ENT1. In human hepatocytes, the involvement of CNT1 and ENT1 in ribavirin uptake was suggested, but the relative contribution of ENT1 to the total ribavirin uptake was more than 80% (Choi et al. 2015). Therefore, ENT1 may play a major role in ribavirin uptake not only in K562 cells but also in various other types of cells.

Because the major side effect of ribavirin is hemolytic anemia, which is induced by the accumulation of ribavirin and its metabolites in erythrocytes, we have been studying the transport mechanism of ribavirin across human erythrocyte membranes (Takano et al. 2010; Yumoto et al. 2010). Using intact erythrocytes and rightside-out membrane vesicles (ROVs) prepared from human erythrocytes, we and others found that ribavirin was transported by ENT1 across human erythrocyte membranes (Jarvis et al. 1998; Yumoto et al. 2010). The kinetics of ribavirin uptake and the sensitivity of the uptake to various ENT inhibitors in K562 cells were similar to those observed in human erythrocyte ROVs. We also examined the interaction of ribavirin with ATP-dependent efflux transporters using inside-out membrane vesicles prepared from human erythrocytes (Yumoto et al. 2010). Ribavirin weakly interacted with multidrug resistance-associated protein 4 (MRP4/ABCC4), an ATP-binding cassette (ABC) efflux transporter, but was not transported by ABC transporters. Similarly, no contribution of other ABC transporters, P-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2), or MRP2 (ABCC2), was detected in MDCKII cells overexpressing them, or in closed circuit dual perfusion experiments with rat term placenta (Karbanova et al. 2019). In this study, we observed that MRP inhibitors as well as ATP depletion did not increase ribavirin accumulation in K562 cells (data not shown). Taken together, ABC efflux transporters would not be involved in ribavirin transport in these cells including K562.

In this study, we measured ribavirin uptake at 23 °C but not at 37 °C, because the uptake at 37 °C was too rapid to observe time-dependent uptake in K562 cells. Ribavirin uptake at 23 °C was much higher than that at ICT (Fig. 1A). Interestingly, however, when the effect of NBMPR on ribavirin uptake was examined at ICT, NBMPR inhibited the uptake in a concentration-dependent manner in K562 cells (data not shown). We have previously examined the functionality of ENT1 in human erythrocyte ROVs, and found that a facilitative transporter ENT1 functions even at very low temperatures near 0 °C (Takano et al. 2010). Similar low-temperature tolerance was observed in D-glucose uptake in ROVs mediated by another facilitative transporter, GLUT1. Karbanova et al. (2019) also examined ribavirin uptake in BeWo cells at 4 °C, and observed a concentration-dependent inhibitory effect of NBMPR on ribavirin uptake. These findings support our conclusion that ribavirin transport is mediated by a facilitative transporter ENT1, which shows low-temperature tolerance, in K562 cells.

ENT1 is a member of the human equilibrative nucleoside transporter family SLC29A, which contains four members ENT1-ENT4 (http://slc.bioparadigms.org/). Among these ENTs, ENT1 is highly sensitive to NBMPR and dipyridamole (Simon M 1986; Ward et al. 2000; Young et al. 2013). In addition, high-affinity NBMPR binding sites were identified in K562 cells (Boleti et al. 1997), further indicating that ENT involved in ribavirin uptake in K562 cells would be mainly ENT1, though possible involvement of other ENT members cannot be denied completely. Predicted topology of ENT1 suggests that it

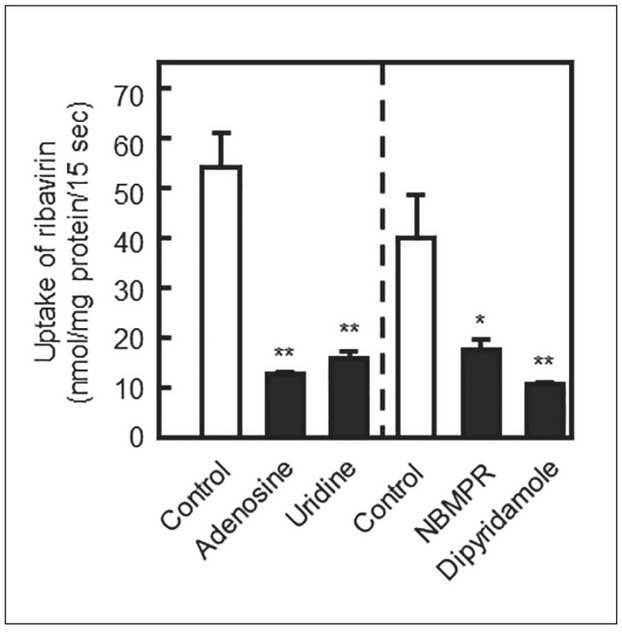

Fig. 7: Effect of ENT substrates and inhibitors on [^{3}H]ribavirin uptake in differentiated K562 cells treated with NaBu. Differentiated K562 cells were incubated with 15 μM [^{3}H]ribavirin in the absence (control, open column) or presence (closed column) of 2 mM adenosine, 2 mM uridine, 10 μM NBMPR, and 10 μM dipyridamole. Each value represents the mean±S.E.M. (n=4). *p< 0.05, **p < 0.01, significantly different from each control.

contains 11 transmembrane domains with a cytoplasmic N-terminus, an extracellular C-terminus, and a large extracellular loop between the first and second transmembrane domains. ENT1 is widely expressed in various mammalian tissues, and has an important physiological role in nucleoside uptake for salvage pathways of nucleotide synthesis. In addition, ENT1 mediates cellular uptake of several drugs that are used for pharmacotherapy of cancer and viral infections (Baldwin et al. 2004; Inoue 2017; Young et al. 2013). Huang et al. (2017) studied the substrate specificity of ENT1 and sensitivity of ENT1-mediated adenosine transport to various drugs using ENT1-incorporated proteoliposomes. They suggested that ENT1 recognized various nucleosides and deoxynucleosides such as adenosine, uridine, thymine, and uracil as substrates. In addition, nucleoside-derived drugs such as cladribine, capecitabine, and ribavirin were shown to inhibit ENT1-mediated adenosine transport. Thus, ENT1 would play physiologically, pathophysiologically, and pharmacologically important roles in many tissues.

It was reported that K562 cells underwent differentiation under certain stimuli (Alitalo 1990; Li et al. 2008; Yu et al. 1989). NaBu can induce G0/G1 arrest and erythroid differentiation in K562 cells, possibly via the upregulation of p18^{INK4C} gene (Li et al. 2008). Erythroid differentiation of K562 cells can also be induced by IMPDH inhibitors including ribavirin and tiazofurin (Yu et al. 1989). For the erythroid differentiation, cationic amino acid transporter 1 (CAT1/SLC7A1) which transports L-arginine and an organic cation transporter OCTN1 (SLC22A4) were suggested to be involved (Nakamura et al. 2007; Shima et al. 2006). In this study, we used NaBu, and erythroid differentiation of K562 cells was confirmed by the change in the color of the cells to red (hemoglobin synthesis) and upregulation of glycophorin A mRNA expression. However, ribavirin uptake activity and sensitivity of the uptake to ENT inhibitors were not different between native and differentiated K562 cells. As described above, we previously reported that ribavirin was transported by ENT1 in mature erythrocyte membranes (Takano et al. 2010; Yumoto et al. 2010). Therefore, ENT1 may be functioning throughout the differentiation of erythroid-lineage cells.

In conclusion, ribavirin uptake into K562 cells was found to be mediated mainly by ENT1, which would be closely associated with its anticancer effect in K562 leukemia cells.

4. Experimental

4.1. Chemicals and reagents

Ham's F-12 Nutrient Mixture (F-12) and fetal bovine serum (FBS) were purchased from MP Biomedicals (Solon, OH, USA). [^{3}H]Ribavirin (6.5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Unlabeled ribavirin, NaBu, and di-n-butyl phthalate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Adenosine, uridine, NBMPR, and dipyridamole were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mineral oil was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals used for experiments were of the highest purity commercially available.

4.2. Cell culture of K562 cells

K562 cells were obtained from RIKEN BioResource Research Center (Tokyo, Japan) and were cultured in F-12 medium with 10% FBS in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were subcultured every 4 days at a density of 1 x 10^5 cells/mL in 25-cm^2 uncoated flask. Medium was changed every 2 days. To induce erythroid differentiation, K562 cells were seeded at a density of 3 x 10^5 cells/mL and were treated with 1 mM NaBu for 4 days.

4.3. Uptake study of [^{3}H]ribavirin in native and differentiated K562 cells

Native and differentiated K562 cells were collected by centrifugation at 500 x g for 5 min and the packed cells were resuspended in HEPES-buffered saline (HBS: 145 mM NaCl, 3 mM KCl, 0.3 mM $CaCl_2$, 0.6 mM $MgCl_2$, 5 mM D-glucose, 5 mM HEPES, pH 7.4) at a density of 1 x 10^7 cells/mL.

To investigate the time- and temperature-dependence of ribavirin uptake, 0.1 mL of cell suspension was quickly mixed with 0.1 mL of HBS containing [^{3}H]ribavirin (final concentration, 15 μM) and was incubated for indicated times at 23 °C or ICT. All the studies were performed after the preincubation of the cells with HBS for 10 min at 23 °C or ICT. To investigate the concentration-dependence of ribavirin uptake, the cells were incubated with various concentrations of unlabeled ribavirin containing 15 μM [^{3}H]ribavirin at 23 °C. To estimate the kinetic parameters for ribavirin uptake, curve fitting was performed using the following equation; $V = V_{max}*[S]/(K_m + [S]) + K_d*[S]$, where V is the ribavirin uptake rate, V_{max} is the maximum uptake rate, K_m is Michaelis-Menten constant, S is the ribavirin concentration, and K_d is diffusion constant, as reported previously (Kawami et al. 2015).

For inhibition studies, the cells were incubated with 15 μM [^{3}H]ribavirin with or without various concentrations of ENT substrates (adenosine and uridine) or ENT inhibitors (NBMPR and dipyridamole) for 15 s at 23 °C. The half-maximal inhibitory concentration (IC_{50}) value was determined with the following Hill equation:

$$V = 100/[1+([I]/IC_{50})^{\gamma}]$$

where V is the ribavirin uptake rate (% of control), [I] is the concentration of each substrate or inhibitor, and γ is the Hill coefficient. The IC_{50} value was determined through curve fitting to the above equation.

To examine the sodium dependence of ribavirin uptake, the cells were incubated with HBS or sodium-free HEPES buffer (145 mM choline chloride, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, 5 mM HEPES, pH 7.4) containing 15 μM [^{3}H]ribavirin for 15 s at 23 °C after preincubation of the cells with HBS or sodium-fee HEPES buffer for 10 min.

After incubation, uptake reaction was terminated by the inhibitor-oil stop method with a slight modification (Takano et al. 2010). Briefly, the reaction mixture (0.15 mL) was added to 0.4 mL of ice-cold stop solution (glucose-free HEPES buffer containing 10 μM NBMPR) layered on top of 0.5 mL ice-cold mixed oil (di-n-butyl phthalate : mineral oil = 19:2) contained in 1.5-mL microcentrifuge tubes. Tubes were immediately centrifuged (10,000 g, 13 s), and the aqueous and mixed oil layers were removed by aspiration and the inside of the tube was wiped dry, leaving a cell pellet at the bottom of the tube. Radioactivity associated with the cell pellet was determined by liquid scintillation counting.

4.4. mRNA expression of ENT1 and glycophorin A in K562 cells

Total RNA was extracted from the cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Laval, QC, Canada), and was reverse transcribed into cDNA using ReverTra Ace (TOYOBO, Osaka, Japan). Real-time PCR was performed with the CFX Connect™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the KAPA SYBR® FAST qPCR Kit (NIPPON Genetics, Tokyo, Japan), according to the manufacturer's instructions. The primer sequences were: human ENT1 sense, 5'-CACCATG-ACAACCAGTCACC-3' and antisense, 5'- GGCGGTTTGTGAAATACTGA-3'; human glycophorin A sense, 5'- CTAACTTCAGGAACCAGCTCA-3' and antisense, 5'-CCTCTTCTGGAGGGTAAACAG-3'; human GAPDH sense, 5'-ACGGGAAGCTTGTCATCAAT-3' and antisense, 5'-TGGACTCCACGAC-GTACTCA-3'. The expression levels of the mRNA were normalized to that of GAPDH mRNA, a housekeeping gene.

4.5. Statistical analysis

All data were expressed as the mean±standard error of mean (S.E.M.). Statistical analysis was performed using the Student's *t*-test, or one-way ANOVA followed by the Tukey-Kramer's test for multiple comparisons. The level of significance was set at *p < 0.05 or **p < 0.01.

Conflicts of interest: None declared.

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