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Effects of cyanidin and its glycosides on the transport of rhodamine 123 in human renal proximal tubular epithelial cell line HK-2

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ABSTRACT

Anthocyans, comprising anthocyanins and their aglycones (anthocyanidins), are a class of flavonoids that are widely distributed in fruits, vegetables and beverages. In this study, we investigated the effects of cyanidin (anthocyanidin), and cyanidin 3-glucoside and cyanidin 3-rutinoside (anthocyanins) on the transport of rhodamine 123, a fluorescent cationic dye often used as a substrate for P-glycoprotein, in human renal proximal tubular cell line HK-2. The steady-state accumulation of rhodamine 123 in HK-2 cells significantly increased in the presence of cyanidin as well as verapamil, a typical P-glycoprotein inhibitor. On the other hand, cyanidin 3-glucoside and cyanidin 3-rutinoside, but not cyanidin, significantly decreased the initial uptake of rhodamine 123 *via* an influx transporter that is inhibited by cimetidine, a pan-inhibitor of organic cation transporters. In contrast to co-incubation with cyanidin 3-glucoside and cyanidin 3-rutinoside, pre-incubation with the two anthocyanins had no effect on the initial uptake of rhodamine 123 in HK-2 cells. These findings suggest that cyanidin, but not its glycosides, inhibits P-glycoprotein-mediated efflux of rhodamine 123, while the two anthocyanins, but not their aglycone, decrease the influx transporter that is involved in the initial uptake of rhodamine 123 in HK-2 cells.

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KEYWORDS: rhodamine 123, P-glycoprotein, organic cation transporter, cyanidin, cyanidin 3-glucoside, cyanidin 3-rutinoside, HK-2 cells.

INTRODUCTION

Anthocyans are a class of natural flavonoids that are widely distributed in fruits, vegetables and beverages. These water-soluble pigments are responsible for the red, blue and purple colors of various plant tissues. Many different anthocyans are found in nature and consist of two types of compounds, anthocyanins and anthocyanidins (aglycones of anthocyanins) [1, 2]. The anthocyanidins most commonly found in flowers and fruits of many plants are cyanidin, malvidin, peonidin, delphinidin, pelargonidin and petunidin. Anthocyans are reported to show various biological effects such as antioxidative, anti-inflammatory, anti-hyperlipidemic, anti-hyperglycemic and antitumor ones [1-4].

Flavonoids have been reported to interact with drug transporters that mediate the cellular uptake and efflux of xenobiotics in various tissues of the body [5, 6]. Drug transporters that are expressed in the small intestine, brain, liver and kidneys are the major determinants of the absorption, distribution and elimination of drugs in the body. Drug transporters are categorized into two main classes, ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters. Several reports suggested that anthocyans affect the transport

that is mediated by ABC transporters such as P-glycoprotein (MDR1/ABCB1) and breast cancer resistance protein (BCRP/ABCG2) [7, 8]. Cyanidin is reported to enhance the accumulation of rhodamine 123 in multidrug-resistant human epidermal carcinoma cell line KB-C2 cells overexpressing P-glycoprotein [7]. In addition, aglycones including cyanidin and pelargonidin have mild inhibitory effects on the ATPase activity of P-glycoprotein, while little effect of their glycosides on the ATPase activity has been observed [8]. Furthermore, twelve anthocyans have been shown to be potential BCRP inhibitors [8]. In addition to efflux transporters, malvidin-3-glucoside inhibits the uptake of bromosulfophtalein *via* bilitranslocase in rat renal basolateral membrane vesicles [9].

Rhodamine 123, a lipophilic cationic fluorescent dye, selectively accumulates in mitochondria based on the membrane potential. Therefore, rhodamine 123 was initially used to stain the mitochondria in living cells. Subsequently, rhodamine 123 was shown to be a substrate for P-glycoprotein and has been widely used to evaluate the transporter activity *via* P-glycoprotein in normal and cancer cells [10-13]. Recently, rhodamine 123 was shown to be a high affinity substrate for human organic cation transporter (OCT) 1 and OCT2, which play an important role in the influx of organic cations into cells [14]. Furthermore, it has been suggested that rhodamine 123 is transported by the organic cation/carnitine transporters OCTN1 and OCTN2 [15]. Thus, the results of an *in vitro* uptake study with rhodamine 123 might reflect the activities of not only efflux transporters such as P-glycoprotein but also influx transporters that are involved in the cellular uptake of cationic xenobiotics, according to the experimental conditions.

In this study, we examined the effects of cyanidin and its glycosides (cyanidin 3-glucoside, cyanidin 3-rutinoside) on the initial uptake and the steady-state accumulation of rhodamine 123 in human renal proximal tubular cell line HK-2.

MATERIALS AND METHODS

Materials

Rhodamine 123 and verapamil hydrochloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cyanidin chloride, cyanidin 3-glucoside and cyanidin 3-rutinoside were obtained from Nagara Science Co., Ltd. (Gifu, Japan). Cimetidine was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals used in the experiments were commercial products of the highest purity available.

Cell culture

HK-2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured as previously described [16, 17]. Briefly, the cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (Nacalai Tesque, Kyoto, Japan), containing 10% fetal bovine serum (FBS), under an atmosphere of 5% CO_2-95% air at 37 °C, and were subcultured every 7 days. The medium was replaced with fresh medium every 2 or 3 days.

Uptake study

After HK-2 cells had been cultured on 24-well tissue culture plates (Iwaki, Tokyo, Japan) for 6 or 7 days, each well was washed and preincubated with phosphate-buffered saline (PBS) (in mM, 137 NaCl, 3 KCl, 8 K_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$) containing 5 mM D-glucose [PBS(G)]. Then, the accumulation of rhodamine 123 (20 μM) was determined. Briefly, PBS(G) buffer containing rhodamine 123 with or without a tested compound was added to each well, and the cells were incubated at 37 °C for the indicated periods. Dimethyl sulfoxide was used as a solvent and the final concentration was less than 0.5%. At the end of the incubation, the uptake buffer was aspirated off and the wells were rinsed rapidly three times with ice-cold PBS buffer. To each well 0.1% Triton X-100 in PBS buffer without $CaCl_2$ or $MgCl_2$ [PBS(-)] was added, and then the cells were scraped off with a cell scraper. The wells were rinsed again to improve the recovery of the cells. The cells were solubilized in 0.1% Triton X-100 in PBS(-) buffer for 30 min at room temperature and then centrifuged at 10,000 rpm for 5 min. The supernatant was used for fluorescence and protein assays. The fluorescence was measured using an EnSpire® Multimode Plate Reader (PerkinElmer, Inc., Waltham, MA) at excitation/emission wavelengths of 485/538 nm. Protein contents were determined by the Bradford method with bovine serum albumin as a standard [18].

The accumulation of rhodamine 123 was normalized as to the protein content of the cells in each well.

Data analysis

Statistically significant differences were determined by means of Student's t-test, or one way or two way analysis of variance with the Tukey's HSD test for post hoc analysis. A p value of less than 0.05 was considered statistically significant.

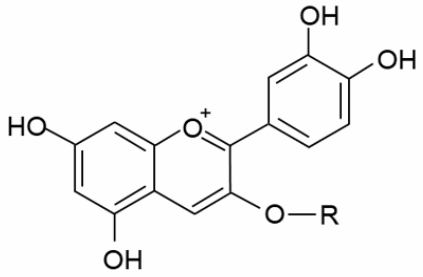
RESULTS

Effect of co-incubation with anthocyans on the time-course of rhodamine 123 uptake in HK-2 cells

Figure 1 shows the chemical structures of cyanidin, cyanidin 3-glucoside and cyanidin 3-rutinoside, which were tested in this study. First, we examined the time-course (at 5, 10, 30 and 120 min) of rhodamine 123 uptake in the absence or presence of each compound in HK-2 cells. Verapamil, an inhibitor of P-glycoprotein, significantly increased the accumulation of rhodamine 123 at 30 and 120 min, but not at 5 and 10 min, in HK-2 cells (Figure 2A). Like verapamil, cyanidin significantly increased the accumulation of rhodamine 123 at 120 min, but not at 5 and 10 min, in HK-2 cells (Figure 2B). In contrast, the two glycosides of cyanidin, cyanidin 3-glucoside and cyanidin 3-rutinoside, significantly inhibited the accumulation of rhodamine 123 in HK-2 cells (Figure 2C and D).

Effect of co-incubation with anthocyans at various concentrations on rhodamine 123 uptake in HK-2 cells

Next we investigated the concentration-dependent effect of anthocyans on the uptake of rhodamine 123 in HK-2 cells. The effects of cyanidin and its glycosides on the influx and efflux of rhodamine 123 were evaluated as the initial uptake of rhodamine 123 in 5 min (Figure 3), and the steady-state accumulation of rhodamine 123 in 120 min (Figure 4), respectively. Figure 3A shows that cimetidine, a pan-inhibitor of organic cation transporters, decreased the initial uptake of rhodamine 123 in a concentration-dependent manner. This finding indicates that the initial uptake of rhodamine 123 is, at least in part, mediated by an organic cation transporter.



	R
Cyanidin	H
Cyanidin 3-glucoside	glucose
Cyanidin 3-rutinoside	rutinose

Figure 1. Chemical structures of cyanidin, cyanidin 3-glucoside and cyanidin 3-rutinoside.

Cyanidin increased the initial uptake of rhodamine 123 in HK-2 cells, though a significant difference was not observed (Figure 3B). In contrast, cyanidin 3-glucoside and cyanidin 3-rutinode inhibited the initial uptake of rhodamine 123 in HK-2 cells in a concentration-dependent manner (Figure 3C and D).

Figure 4A shows that verapamil enhanced the steady-state accumulation of rhodamine 123 in a concentration-dependent manner. This finding indicates that the steady-state accumulation of rhodamine 123 is modulated by the inhibition of the efflux transporter P-glycoprotein (Figure 4A). Cyanidin also enhanced the steady-state accumulation of rhodamine 123 in a concentration-dependent manner (Figure 4B). In contrast, cyanidin 3-glucoside decreased the steady-state accumulation of rhodamine 123 in a concentration-dependent manner (Figure 4C). In the case of cyanidin 3-rutinoside, weak inhibition of the steady-state accumulation of rhodamine 123 was observed at the maximum concentration, though it was not significant (Figure 4D).

Effect of pre-incubation with anthocyanins on the time-course of rhodamine 123 uptake in HK-2 cells

To clarify whether or not the inhibitory effects of cyanidin 3-glucoside and cyanidin 3-rutinoside on rhodamine 123 uptake are due to competitive

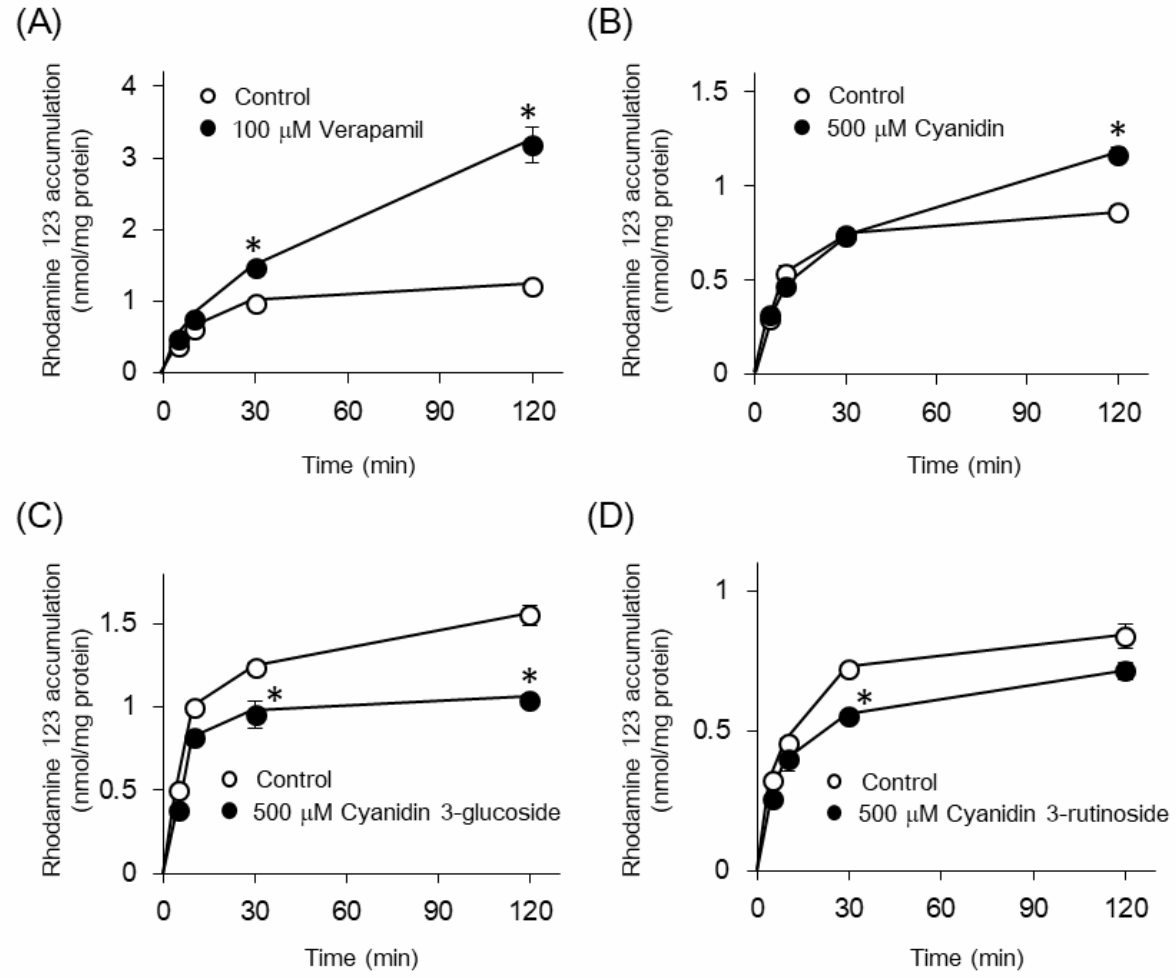


Figure 2. Effect of co-incubation with anthocyans on the time-course of rhodamine 123 uptake in HK-2 cells. The transport of rhodamine 123 was measured at 37 °C at 5, 10, 30 and 120 min in the buffer in the absence (open symbols) or presence (close symbols) of verapamil (100 μM) (A), cyanidin (Cya, 500 μM) (B), cyanidin 3-glucoside (C3G, 500 μM) (C), and cyanidin 3-rutinoside (C3R, 500 μM) (D). Each symbol represents the mean ± S.E. for three to four monolayers. *$p < 0.05$, significantly different from the value for the control at each time.

inhibition of the influx transporter, we examined the effect of pre-incubation of the two anthocyanins on rhodamine 123 transport in HK-2 cells. As shown in Figure 5, the pre-treatment with cyanidin 3-glucoside and cyanidin 3-rutinoside had no significant effect on rhodamine 123 uptake in HK-2 cells at any incubation time tested. The results indicate that the inhibition of rhodamine 123 uptake in the presence of cyanidin 3-glucoside and cyanidin 3-rutinoside is due to competitive inhibition, but not due to decreased expression and conformation changes of the influx transporter protein that is involved in the uptake of rhodamine 123 in HK-2 cells.

DISCUSSION

In this study, the uptake of rhodamine 123 in HK-2 cells was measured after incubation periods of 5, 10, 30 and 120 min. The accumulation of rhodamine 123 in the absence of a test compound (control) linearly increased up to 10 min, and it remained stable at 30 min or longer, reaching a steady state. Therefore, we considered that the uptake of rhodamine 123 at 5 min reflects the influx transporter activity, and the accumulation of rhodamine 123 at 120 min reflects both the influx and efflux transporter activities.

The accumulation of rhodamine 123 at 120 min was significantly enhanced by cyanidin as well as

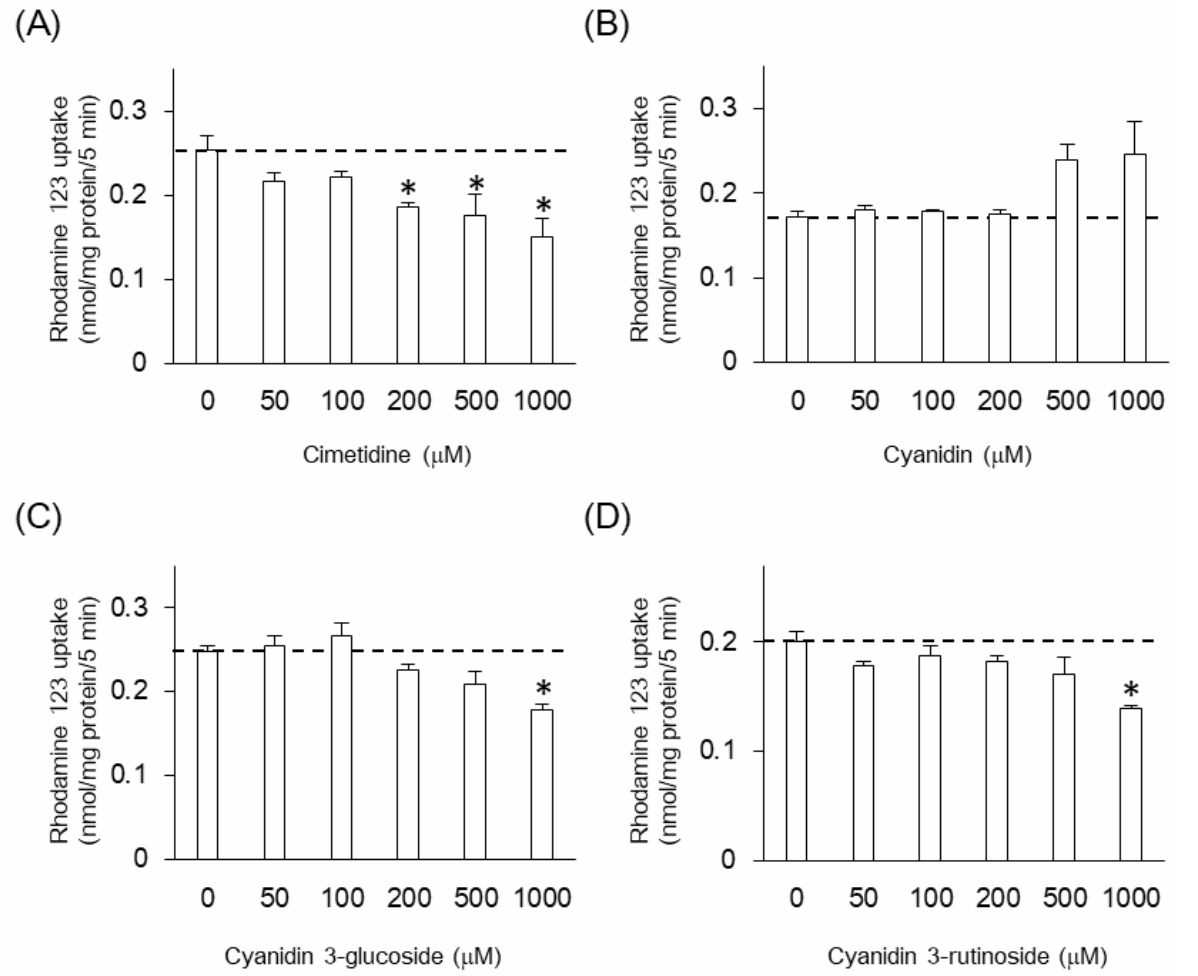


Figure 3. Effect of co-incubation with anthocyans on the initial uptake of rhodamine 123 uptake in HK-2 cells. The transport of rhodamine 123 was measured at 37 °C at 5 min in the buffer in the absence or presence of cimetidine (A), cyanidin (B), cyanidin 3-glucoside (C), and cyanidin 3-rutinoside (D) at concentrations of 50, 100, 200, 500 and 1000 μM. Each column represents the mean ± S.E. for three to four monolayers. *$p < 0.05$, significantly different from the value for the control (0 μM).

by a typical P-glycoprotein inhibitor verapamil. This finding suggests that cyanidin inhibits the efflux of rhodamine 123 *via* P-glycoprotein expressed in HK-2 cells, which is in agreement with the decreased P-glycoprotein activity caused by cyanidin shown in the previous report [7]. In contrast, the two cyanidin glycosides, cyanidin 3-glucoside and cyanidin 3-rutinoside, decreased the accumulation of rhodamine 123 due to the reduced activity of the influx transporter, as described below. Though this present finding does not necessarily exclude the possibility that cyanidin 3-glucoside and cyanidin 3-rutinoside modulate the efflux transporter activity, it is unlikely that the two glycosides inhibit the P-glycoprotein activity of rhodamine 123, taking into consideration the previous findings that anthocyanins including cyanidin 3-glucoside and cyanidin 3-rutinoside had no effect on MDR1 ATPase activity [8].

The uptake of rhodamine 123 at 5 min was significantly inhibited by cyanidin 3-glucoside and cyanidin 3-rutinoside, but not cyanidin. This finding suggests that anthocyanins have an inhibitory effect on the influx transporter that is involved in the cellular uptake of rhodamine 123 in HK-2 cells. To clarify the involvement of organic cation transporter(s), we examined the dose-dependent effect of the pan-organic cation

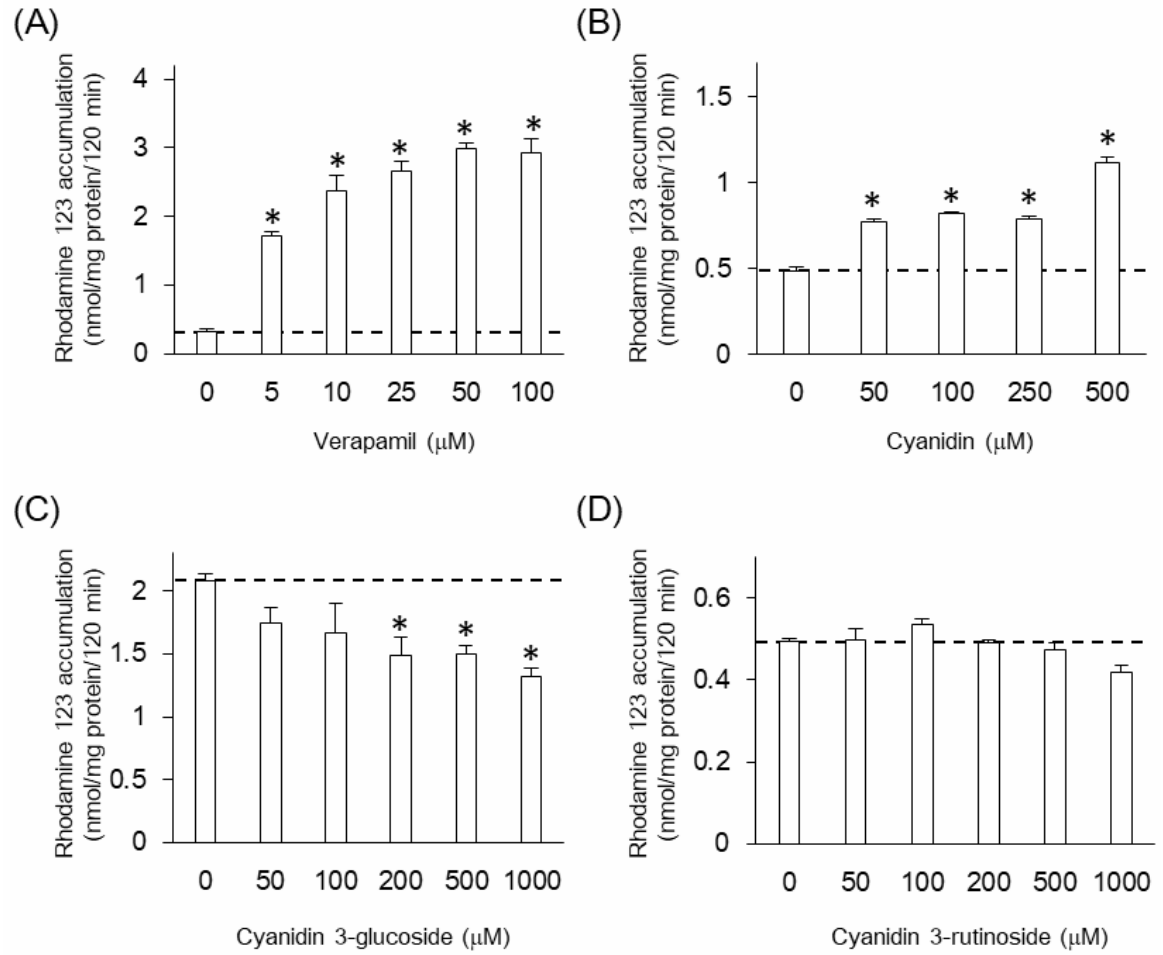


Figure 4. Effect of co-incubation with anthocyans on the steady-state accumulation of rhodamine 123 in HK-2 cells. The transport of rhodamine 123 was measured at 37 °C at 120 min in the buffer in the absence or presence of cimetidine (A), cyanidin (B), cyanidin 3-glucoside (C), and cyanidin 3-rutinoside (D) at concentrations of 50, 100, 200, 500 and 1000 μM. Each column represents the mean ± S.E. for three to four monolayers. *$p < 0.05$, significantly different from the value for the control (0 μM).

transporter inhibitor cimetidine on the initial uptake of rhodamine 123 in HK-2 cells. Cimetidine decreased the uptake of rhodamine 123 at 5 min in a concentration-dependent manner, but the reducing effect of cimetidine was only about 40% of the control level even at the maximum concentration of 1 mM. According to a kinetic study based on the Michaelis-Menten equation, we found that the initial uptake of rhodamine 123 at a concentration of 20 μM comprised 49.6% of transporter-mediated transport and 50.4% of simple diffusion-mediated transport [Micahelis constant (Km), 27.9 μM; maximum velocity (Vmax) for rhodamine 123 uptake, 0.240 nmol/mg protein/5 min; diffusion coefficient, 5.11 x 10^{-3} mL/mg protein/5 min] (Figure 6). Therefore, it is likely that cyanidin 3-glucoside and cyanidin 3-rutinoside relatively firmly decrease the transporter-mediated uptake of rhodamine 123 in HK-2 cells.

So far, rhodamine 123 has been reported to be a substrate for various influx transporters. Rhodamine 123 is suggested to be a high-affinity substrate for OCT1 and OCT2 [14]. However, little mRNA expression of SLC22A2 (OCT2) in HK-2 cells has been observed compared to that in normal human renal cortex samples [19]. In addition, verapamil, a typical inhibitor of OCT1/2 as well as P-glycoprotein, showed no significant inhibition of the initial uptake of rhodamine 123 in HK-2 cells.

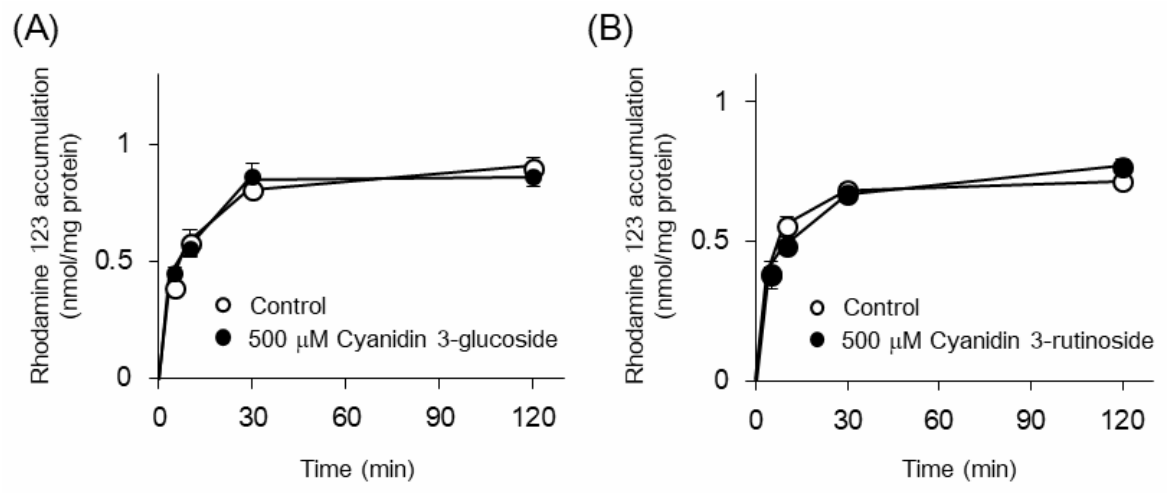


Figure 5. Effect of pre-incubation with anthocyanins on the time-course of rhodamine 123 uptake in HK-2 cells. The cells were pretreated with cyanidin 3-glucoside (C3G, 500 μM) (A) and cyanidin 3-rutinoside (C3R, 500 μM) (B) for 30 min at 37 °C, and then the transport of rhodamine 123 was measured at 37 °C at 5, 10, 30 and 120 min. Each symbol represents the mean ± S.E. for three monolayers.

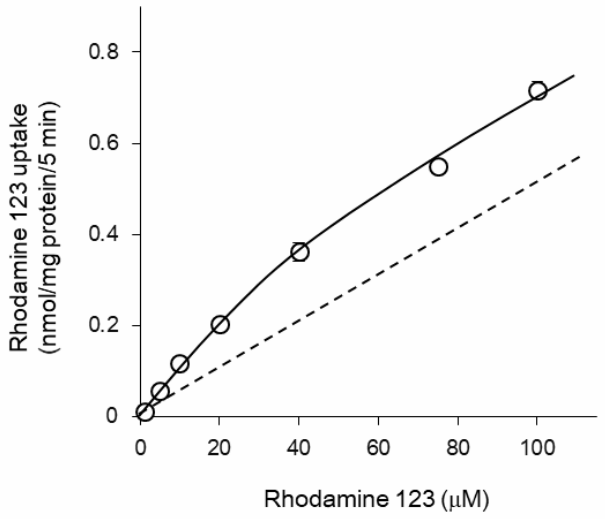


Figure 6. Concentration dependence of rhodamine 123 uptake in HK-2 cells. Rhodamine 123 uptake for 5 min at concentrations between 1 and 100 μM was determined at 37 °C. Each point represents the mean ± S.E. for three monolayers. The uptake of rhodamine 123 was evaluated kinetically using non-linear least-squares regression analysis following the Michaelis-Menten equation with a linear non-saturable component. The dashed line represents the non-saturable uptake of rhodamine 123. The estimated kinetic parameters were Micahelis constant (Km), 27.9 μM; maximum velocity (Vmax) for rhodamine 123 uptake, 0.240 nmol/mg protein/5 min; diffusion coefficient (Kd), 5.11 x 10^{-3} mL/mg protein/5 min.

The lack of an inhibitory effect of verapamil on the initial uptake of rhodamine 123 rules out the involvement of PMAT (plasma membrane monoamine transporter), which is strongly inhibited by verapamil [20]. Recently, it was shown that rhodamine 123 is transported by the apical organic cation transporters OCTN1/2 in Calu-3 human lung epithelial cells [15]. In addition, cimetidine is reported to be an inhibitor of OCTN1/2 [21]. In this study, the uptake study was performed using cells cultured on culture plates. Therefore, it remains unclear at present whether the influx of rhodamine 123 in HK-2 cells occurs from the apical surface or the basal surface. Further studies are needed to identify the influx transporter that plays an important role in the uptake of rhodamine 123 and is inhibited by the anthocyanins in HK-2 cells.

It is reported that treatment with some anthocyans modulates the mRNA and protein expression of organic anion transporting polypeptides OATP1B1 and OATP1B3 in human hepatocytes [22]. To confirm whether or not the inhibitory effects of cyanidin 3-glucoside and cyanidin 3-rutinoside on the initial uptake of rhodamine 123 are due to a change in the expression of the influx transporter, the effect of pre-incubation of the two anthocyanins was investigated. In contrast to the results of

co-incubation, no significant effect on the rhodamine 123 uptake was observed in HK-2 cells pretreated with the two anthocyanins. Therefore, it is likely that cyanidin 3-glucoside and cyanidin 3-rutinoside directly inhibit the uptake of rhodamine 123 in HK-2 cells.

CONCLUSION

In conclusion, we suggest that cyanidin, but not cyanidin 3-glucoside or cyanidin 3-rutinoside, inhibits the efflux of rhodamine 123 *via* P-glycoprotein in HK-2 cells. In contrast, it is likely that cyanidin 3-glucoside and cyanidin 3-rutinoside, but not cyanidin, decrease the influx transporter that is responsible for rhodamine 123 uptake in HK-2 cells.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest to declare.

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