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Contribution of an unidentified sodium-dependent nucleoside transport system to the uptake and cytotoxicity of anthracycline in mouse M5076 ovarian sarcoma cells

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ABSTRACT

In the present study, we investigated whether an unidentified system for Na^+-dependent nucleoside transport is expressed by mouse M5076 ovarian sarcoma cells, besides concentrative nucleoside transporter 2 ($CNT2_M$), and is involved in the uptake and cytotoxicity of anthracyclines. In a transport assay involving $CNT2_M$-transfectants, $CNT2_M$ was found to transport [^{3}H]cytidine in a Na^+-dependent manner, and 500 μM cytidine completely inhibited the Na^+-dependent uptake of [^{3}H]uridine via the transporter. In contrast, the Na^+-dependent [^{3}H]uridine uptake by M5076 cells decreased with 500 μM cytidine only to 70% of the control level. Furthermore, transfection of $CNT2_M$-specific siRNAs into M5076 cells resulted in a reduction in the Na^+-dependent uptake of [^{3}H]uridine by only 23%, although the expression of $CNT2_M$ mRNA and Na^+-dependent uptake of [^{3}H]cytidine disappeared in the cells. The uptake of pirarubicin (THP), an anthracycline, by M5076 cells requiring extracellular Na^+ was significantly inhibited by 500 μM uridine, but not 500 μM cytidine. The Na^+-dependent and cytidine-insensitive uptake of [^{3}H]uridine and the that of THP by M5076 cells significantly increased on cotreatment with both cholate and taurocholate, and the enhancement of THP uptake by the bile acids was reversed by cotreatment with 500 μM uridine. Furthermore, the cytotoxicity of THP and doxorubicin, which were previously reported to be taken up via the same transporter, toward M5076 cells was enhanced by cotreatment with both the bile acids. Therefore, it was indicated that an unidentified Na^+-dependent transport system for nucleosides is expressed by M5076 cells, and contributes to the uptake and cytotoxicity of the anthracyclines.

1. Introduction

Anthracycline antibiotics are the most active chemotherapeutic agents for a variety of malignancies [1–3]. However, resistance to anthracyclines is one of the serious problems restricting their clinical usefulness [4]. We previously reported that mouse Ehrlich ascite carcinoma cells were sensitive to both of pirarubicin (THP) and doxorubicin (DOX), but that M5076 ovarian sarcoma cells exhibited de novo resistance to DOX, but not to THP, which was due to extremely low

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expression of their target enzyme topoisomerase II [5] in M5076 cells [6]. Accordingly, we have investigated relationship of the transport characteristics and cytotoxicity of these anthracyclines between both the types of cell, in order to establish a strategy for overcoming the de novo resistance. The cytotoxicity of THP and DOX toward Ehrlich and M5076 cells depended on the uptake efficacy via the same transporter, probably a Na^+-dependent concentrative nucleoside transporter (CNT), and was not affected by their efflux efficacy [6–8]. Therefore, identification and functional characterization of the CNT contributing to the uptake of anthracyclines are thought to be extremely important.

NTs play key roles in the uptake of precursors for nucleotide synthesis [9], and are also required for efficient cellular uptake of hydrophilic anticancer and antiviral drugs [9,10]. Mammalian cells possess multiple NTs that are either equilibrative or concentrative, of which six isoforms have been molecularly identified [11–13]. CNTs require an inwardly directed Na^+-gradient as a driving force and are classified into three isoforms based on their substrate specificity [11,12]. CNT1 and CNT2 can transport both uridine and adenosine, but prefer pyrimidine and purine nucleosides, respectively, while CNT3 is broad selective. On the other hand, several kinetic studies have indicated that a molecularly unidentified CNT isoform exists in the plasma membranes of mammalian cells [9,14].

Recently, we reported that mRNA for CNT2, but not CNT1 and CNT3, was expressed in M5076 cells, in which the Na^+-dependent uptake activity for uridine, a typical substrate of NTs, was greater than in Ehrlich cells [7,8]. However, the THP uptake by the Cos-7 cells transfected with cDNA of CNT2 isolated from M5076 cells ($CNT2_M$) was almost the same as that by mock cells [8]. Furthermore, there were some differences in the sensitivity of purine nucleosides and THP as the inhibitors to [^{3}H]uridine uptake between M5076 and CNT2-transfected Cos-7 cells [8]. In this study, therefore, we investigated the transport characteristics of nucleosides and THP in M5076 cells with reduced expression of $CNT2_M$ by transfecting small interfering RNA (siRNA), and in $CNT2_M$-overexpressing Cos-7 cells (Cos-7/$CNT2_M$), in order to clarify whether or not an unidentified system for the Na^+-dependent transport of nucleosides is present in M5076 cells, and contributes to the uptake and cytotoxicity of THP in the cells.

2. Materials and methods

2.1. Chemicals

Pure THP and tetrahydropyranyldoxorubicinol (internal standard for the HPLC assay) were gifts from Meiji Seika Kaisha (Tokyo, Japan). Nitrobenzylmercaptopurine riboside (NBMPR), 2-deoxy-D-glucose (DOG), and alanylalanine were purchased from Sigma Chemical Co. (MO, USA), and uridine, adenosine, inosine, thymidine, cytidine and sodium azide (AZ) were from Wako Pure Chemical Ind. (Osaka, Japan). [^{3}H]Uridine (40 Ci/mmol) and [^{3}H]cytidine (30 Ci/mmol) were obtained from American Radiolabeled Chemicals Inc. (MO, USA). All other reagents were of commercial or analytical grade requiring no further purification.

2.2. Cell culture

Cos-7 cells were maintained in Dulbecco's modified Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan) containing 5.6 mM glucose and 10% fetal bovine serum (MP Biochemicals, LLC, CA, USA) at 37 °C under a humidified atmosphere of 5% CO_2 in air. M5076 cells were isolated and purified by washing with an appropriate buffer (pH 7.4), after they had been grown in the cavum abdominis of C57BL/6 male mice (Japan SLC Inc., Hamamatsu, Japan) aged 5–7 weeks. The density and viability (>90%) of cells were determined by means of the trypan blue exclusion test.

2.3. Generation of $CNT2_M$-transfectants

As described previously [8], a cDNA of $CNT2_M$ subcloned into pCI-neo expression vector (Promega Co., WI, USA) was introduced into Cos-7 cells using COATSOME EL-01-D (NOF Co., Tokyo, Japan). The peptide encoded by $CNT2_M$ differed from that by the previously reported mouse CNT2 in five substitutions, that is, Arg 14 Tyr, Gly 19 Cyt, Tyr 92 Ala, Ile 154 Val and Tyr 336 Val.

2.4. RNA interference

Sense and antisense siRNAs were synthesized and annealed by NIPPON EGT Co. (Toyama, Japan). An siRNA duplex with the sequences 5′-GGAGUCAAGUUCUUCAUAAtt-3′ (sense) and 5′-UUAUGAAGAACUUGAC-UCCtt-3′ (antisense) was used. M5076 cells were cultured in Eagle's MEM supplemented with 10% fetal bovine serum for 72 h, and then were transfected with siRNA (132 ng of RNA/cm^2) using LipofectAMINE 2000 (Invitrogen, CA, USA) and OPTI-MEM reduced serum medium (Invitrogen). After 24 h culture, the cells were used for the expression and uptake assay. Control cells were treated with LipofectAMINE 2000 and OPTI-MEM reduced serum medium without siRNA.

2.5. RT-PCR and real time quantitative RT-PCR

Total RNA was extracted with Sepasol RNA-I super (Nacalai, Kyoto, Japan), and then purified with a GenElute™ Mammalian Total RNA kit (Sigma Chemical Co.) according to the manufacturer's instruction manual. Total RNA was reverse transcribed into cDNA by means of Oligo-T priming and Moloney murine leukemia virus reverse transcriptase, and then the cDNA was PCR-amplified at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s with rTaq DNA polymerase (Takara, Shiga, Japan). $CNT2_M$ was amplified with 5′ primer 5′-TTTGGTGATACACTGGTCC-3′ and 3′ primer 5′-CCTGACCA-CAATCTTGCAC-3′ to produce a 957 bp product. β-Actin, as an internal standard, was amplified with 5′ primer 5′-GTGAC-GAGGCCCAGAG-CAAGAG-3′ and 3′ primer 5′-AGGGGCCG-GACTCATCGTACTC-3′ to produce a 939 bp product.

The real time quantitative PCR was performed with an ABI PRISM 7500 Real Time PCR System using SYBR® Premix EX Taq™ (Takara) according to the manufacture's instructions. The primer set for $CNT2_M$ was as follows: sense, 5′-TTTTCCCTCTGTGGATTTGCC-3′ and antisense, 5′-CCTGAC-CACAATCTTGCAC-3′, and that of β-actin as follows: sense, 5′-

AGGTCATCACTATTGGCAACGA-3′ and antisense, 5′-CACTTCATGATGGAATTGAATGTAGTT-3′.

2.6. Uptake assay

Assaying of the uptake by transfectants was performed by the modified method of Nagasawa et al. [15]. The uptake reaction was initiated by adding the indicated concentrations of nucleoside plus ^{3}H-labeled nucleoside (1.7 μCi/mL) to the cells pretreated with 10 μM NBMPR in appropriate buffer (pH 7.4). After appropriate time intervals, the reaction was terminated by the addition of an excess volume of ice-cold choline buffer (pH 7.4) containing 1 mM non-radioactive uridine and 10 μM NBMPR.

The uptake experiment involving M5076 cells was performed by the modified method of Nagasawa et al. [16]. The reaction was initiated by the addition of the indicated concentrations of THP or nucleoside plus ^{3}H-labeled nucleoside (1.7 μCi/mL) to NBMPR-pretreated cell suspension (final cell density: 5×10^6 cells/mL, pH 7.4). After appropriate time intervals, the reaction was terminated by transferring the reaction mixture to Na^+-free buffer (pH 7.4) layered over a cushion of silicone oil (sp. gr. 1.050)/mineral oil (sp. gr. 0.845–0.905) (21:4, v/v), followed by centrifugation for 1 min at 15,000 rpm. In the case of [^{3}H]uridine uptake, to terminate the reaction completely, 1 mM unlabeled uridine and 10 μM NBMPR were added to choline buffer layered over the cushion of oil.

The intracellular concentrations of [^{3}H]nucleoside and THP were determined with a liquid scintillation counter and HPLC, respectively, following the methods of Nagasawa et al. [15,16]. Protein concentrations were determined by the method of Bradford [17] with bovine serum albumin (Sigma Chemical Co.) as the standard.

2.7. Cytotoxicity assay

The in vitro sensitivity of M5076 cells to anthracyclines was assessed by means of the tetrazolium dye (MTT) assay as described previously [16]. Furthermore, we confirmed that the decrease in cell viability observed in the MTT assay was not caused by mitochondria dysfunction, by comparison with the results obtained on the trypane blue exclusion test.

2.8. Statistical analysis

The data are expressed as means ± S.E. or means ± S.E.M. The Michaelis constant (K_m) and maximal transport rate (V_{max}) were calculated on the basis of the Eadie–Hofstee equation. Comparisons between two and more groups were performed by means of Student's unpaired *t*-test and analysis of variance (ANOVA, followed by Fischer's PLSD), respectively, differences with a p value of 0.05 or less being considered statistically significant.

3. Results

3.1. Effects of pyrimidine nucleosides on [^{3}H]uridine uptake mediated by CNT2$_M$

The effects of pyrimidine nucleosides on [^{3}H]uridine uptake via CNT2$_M$ were examined (Fig. 1A). The uptake of [^{3}H]uridine mediated by CNT2$_M$ was significantly reduced by 500 μM

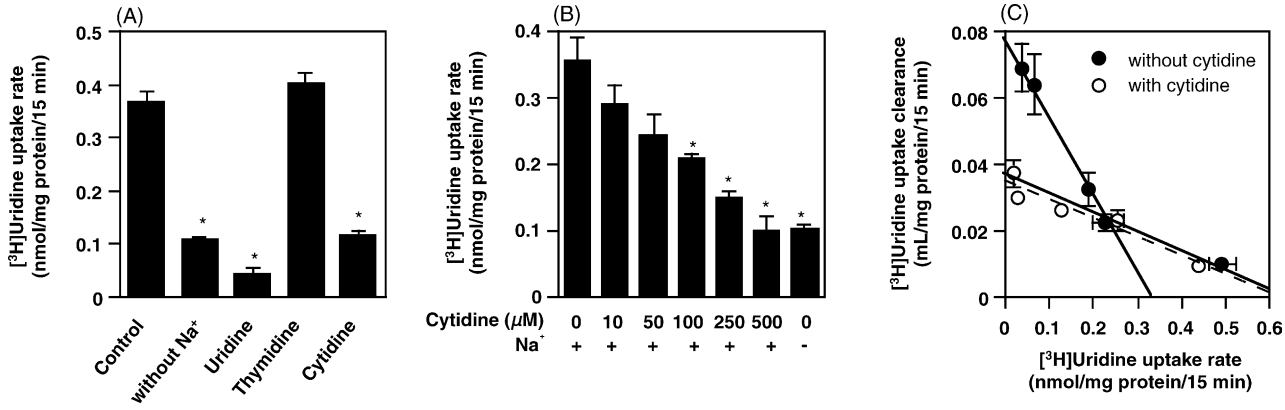

Fig. 1 – Effects of pyrimidine nucleosides on [^{3}H]uridine uptake mediated by CNT2$_M$. (A) The cells were incubated with 5 μM [^{3}H]uridine in HBSS or choline-replaced HBSS containing 10 μM NBMPR in the presence or absence of 500 μM pyrimidine nucleoside for 15 min at 37 °C. The net uptake via CNT2$_M$ was calculated by subtracting the uptake by mock cells from that by Cos-7/CNT2$_M$. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.001$, significantly different from control. (B) The cells were incubated with 5 μM [^{3}H]uridine in HBSS or choline-replaced HBSS containing 10 μM NBMPR and the indicated concentrations of cytidine for 15 min at 37 °C. The net uptake via CNT2$_M$ was calculated by subtracting the uptake by mock cells from that by Cos-7/CNT2$_M$. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.001$, significantly different from group with Na^+ in the absence of cytidine. (C) The cells were incubated with 0.5–50 μM [^{3}H]uridine in HBSS containing 10 μM NBMPR in the presence or absence of 100 μM cytidine for 15 min at 37 °C. The net uptake via CNT2$_M$ was calculated by subtracting the uptake by mock cells from that by Cos-7/CNT2$_M$. Each point represents the mean ± S.E.M. for three experiments. The dotted line showed the low-affinity component of [^{3}H]uridine uptake mediated by CNT2$_M$ without cytidine.

Table 1 – Kinetic constants for [^{3}H]uridine and [^{3}H]cytidine uptake mediated by CNT2$_M$

	K_m (μM)	V_{max} (nmol/mg protein per 15 min)	K_i (μM)
Uridine uptake			
Cytidine (–)-high affinity	4.50 ± 1.19	0.339 ± 0.0571	–
Cytidine (–)-low affinity	17.4 ± 3.22	0.661 ± 0.0469	–
Cytidine (+)	18.5 ± 2.13	0.624 ± 0.0343	66.9 ± 8.02
Cytidine uptake	254 ± 25.9	10.2 ± 0.878	–

The kinetic constants for [^{3}H]uridine and [^{3}H]cytidine uptake were calculated with the Eadie–Hofstee equation using the data in Figs. 1C and 2B, respectively.

cytidine as well as uridine, but thymidine had no effect on the uptake. As depicted in Fig. 1B, the inhibition of CNT2$_M$-mediated uptake of [^{3}H]uridine by cytidine was dose-dependent, and 500 μM cytidine inhibited the [^{3}H]uridine uptake to the same level as that without extracellular Na^+. The Eadie–Hofstee plots of [^{3}H]uridine uptake mediated by CNT2$_M$ in the presence or absence of cytidine are shown in Fig. 1C. The plot of the uptake without cytidine was biphasic, and 100 μM cytidine inhibited the uptake of low, but not high, concentrations of [^{3}H]uridine mediated by CNT2$_M$. The uptake of high concentrations of [^{3}H]uridine via CNT2$_M$ was significantly reduced by an excess amount of uridine, but not cytidine (data not shown). As shown in Table 1, the kinetic constants for the low-affinity component of uptake of [^{3}H]uridine mediated by CNT2$_M$ without cytidine were similar to those for the [^{3}H]uridine uptake with cytidine.

3.2. *Characteristics of [^{3}H]cytidine transport via CNT2$_M$*

Time profiles of [^{3}H]cytidine uptake by Cos-7/CNT2$_M$ and mock cells are shown in Fig. 2A. The transfection of CNT2$_M$ into Cos-7 cells resulted in an increase in [^{3}H]cytidine uptake (Fig. 2A). The uptake of [^{3}H]cytidine by Cos-7/CNT2$_M$ and mock cells was not affected by 3,4,5,6-tetrahydrouridine, typical inhibitor of cytidine deaminase (data not shown). In contrast, there was no difference in [^{3}H]tymidine uptake between Cos-7/CNT2$_M$ and mock cells (data not shown). The [^{3}H]cytidine uptake via CNT2$_M$ showed a clear concentration-dependent profile, K_m = 254 ± 25.9 μM and V_{max} = 10.2 ± 0.878 nmol/mg protein per 15 min (Table 1). The [^{3}H]cytidine uptake mediated by CNT2$_M$ depended on extracellular Na^+, and was significantly inhibited by 500 μM cytidine and uridine (Fig. 2C).

[^{3}H]Cytidine uptake by M5076 cells was characterized. As depicted in Fig. 3A, almost of [^{3}H]cytidine uptake by M5076 cells depended on extracellular Na^+, and the uptake was significantly reduced by cytidine dose-dependently. We further performed the uptake assay using M5076 cells transfected with siRNA specific to CNT2$_M$. On RT-PCR analysis, a PCR product of the expected mobility for CNT2$_M$, but not β-actin, observed in M5076 cells was disappeared by transfection with the siRNA (Fig. 3B). Furthermore, when we examined the amount of CNT2$_M$ mRNA by real-time quantitative PCR, mRNA for CNT2$_M$ was detected in M5076 cells, but not in the siRNA-transfected cells, although mRNA for β-actin was expressed at the same level in the two types of cell (Fig. 3C). The uptake of [^{3}H]cytidine by M5076 cells, but not the siRNA-transfected ones, depended on extracellular Na^+, and was significantly decreased by the addition of 500 μM cytidine (Fig. 3D).

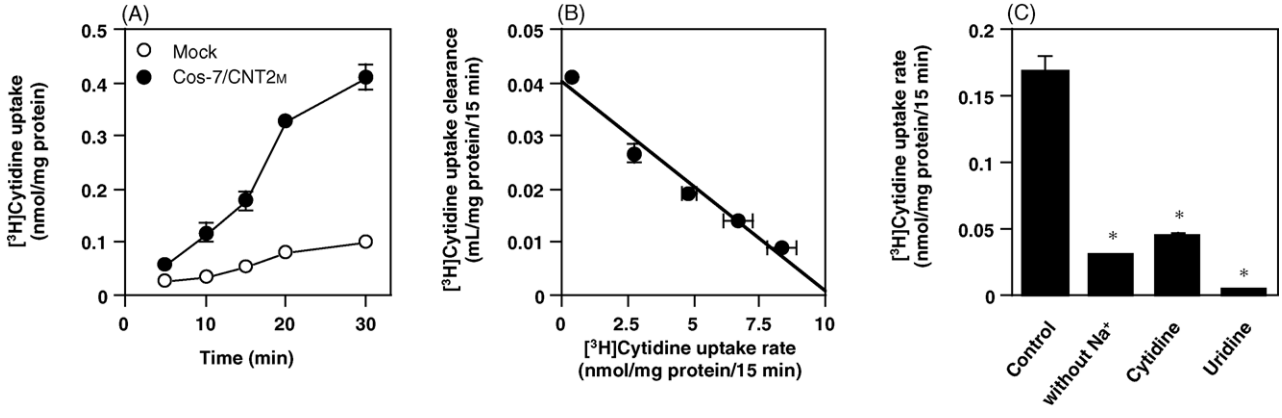

Fig. 2 – Characteristics of [^{3}H]cytidine uptake mediated by CNT2$_M$. (A) Cos-7/CNT2$_M$ and mock cells were incubated with 5 μM [^{3}H]cytidine in HBSS containing 10 μM NBMPR for the indicated times at 37 °C. Each point represents the mean ± S.E. for three experiments. (B) The cells were incubated with 10–1000 μM [^{3}H]cytidine in HBSS containing 10 μM NBMPR for 15 min at 37 °C. The net uptake via CNT2$_M$ was calculated by subtracting the uptake by mock cells from that by Cos-7/CNT2$_M$. Each point represents the mean ± S.E.M. for three experiments. (C) The cells were incubated with 5 μM [^{3}H]cytidine in HBSS or choline-replaced HBSS containing 10 μM NBMPR in the presence or absence of 500 μM of the indicated nucleosides for 15 min at 37 °C. The net uptake via CNT2$_M$ was calculated by subtracting the uptake by mock cells from that by Cos-7/CNT2$_M$. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.001$, significantly different from control.

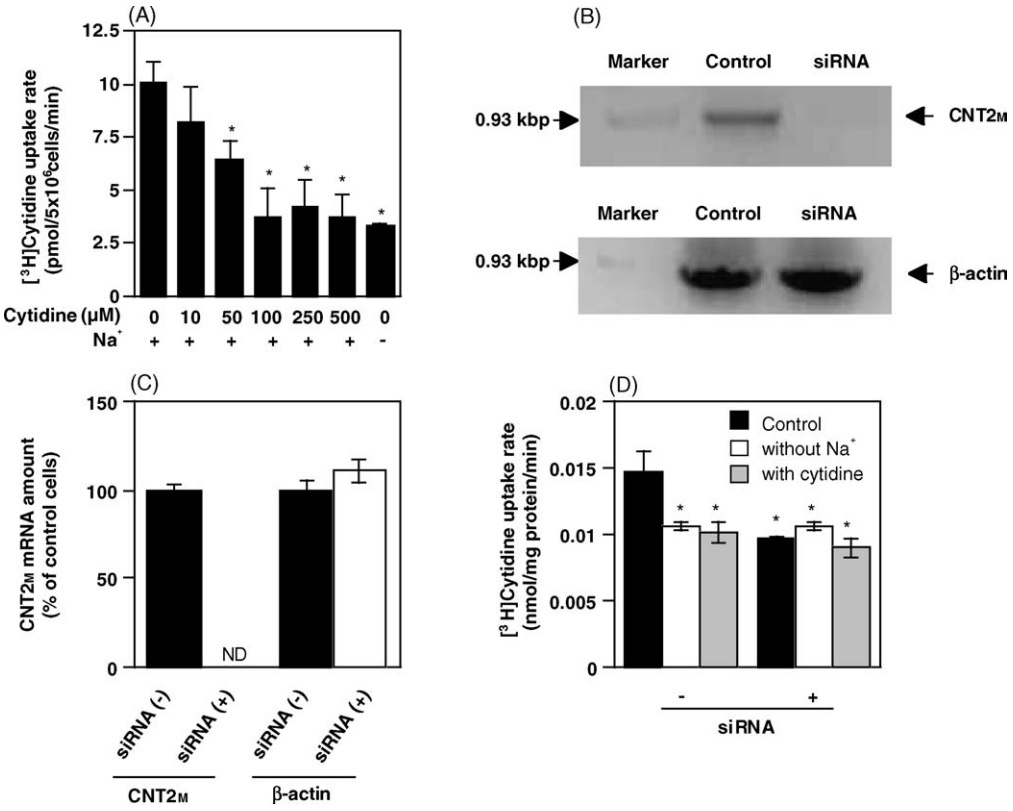

Fig. 3 – Characteristics of [^{3}H]cytidine uptake by M5076 cells. (A) The cells were incubated with 5 μM [^{3}H]cytidine in HBSS or choline-replaced HBSS containing 10 μM NBMPR and the indicated concentrations of cytidine for 1 min at 37 °C. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, significantly different from group with extracellular Na^+ in the absence of cytidine. (B and C) Cells were transfected with $CNT2_M$-specific siRNA, and then the expression of mRNA for $CNT2_M$ was analyzed by semi-quantitative RT-PCR and real time quantitative RT-PCR, respectively. β-Actin was used as an internal control. ND, not detected. (D) The cells were incubated with 5 μM [^{3}H]cytidine in HBSS or choline-replaced HBSS containing 10 μM NBMPR in the presence or absence of 500 μM cytidine for 1 min at 37 °C. Each bar represents the mean ± SE for three experiments. $^*p < 0.05$, significantly different from group with extracellular Na^+ in the absence of cytidine and transfection with siRNA.

3.3. *Characteristics of [^{3}H]uridine transport in M5076 cells*

The effect of cytidine on [^{3}H]uridine uptake by M5076 cells was examined. The uptake of [^{3}H]uridine by M5076 cells was significantly inhibited by cytidine in a concentration-dependent manner, but the Na^+-dependent uptake of [^{3}H]uridine by the cells was reduced by 500 μM cytidine to only 67.2% of the control level (Fig. 4A). The [^{3}H]uridine uptake by M5076 cells in the presence of 500 μM cytidine was extracellular Na^+- and temperature-dependent, and was significantly inhibited by 500 μM uridine (Fig. 4B). Furthermore, despite transfection with $CNT2_M$-specific siRNA, Na^+-dependent uptake of [^{3}H]uridine by the cells was decreased only, but significantly, to 80% (Fig. 4C).

Next, the effects of substrate-inhibitors for various transporters on the uptake of [^{3}H]uridine by M5076 cells and $CNT2_M$ were examined. None of the inhibitors had any effect on the uptake of [^{3}H]uridine by M5076 cells (Fig. 5A). On the other hand, the uptake of [^{3}H]uridine via $CNT2_M$ was significantly inhibited by taurocholate (Fig. 5B). Unexpectedly, cholate significantly increased the uptake of [^{3}H]uridine by M5076 cells with extracellular Na^+ in the presence and absence of 500 μM cytidine, but the uptake mediated by $CNT2_M$ was not affected by this bile acid (Figs. 5 and 6). The [^{3}H]uridine uptake by M5076 cells in the presence, but not the absence, of 500 μM cytidine was significantly increased by the addition of taurocholate (Fig. 6), which was probably due to inhibition of [^{3}H]uridine uptake via $CNT2_M$ by it.

3.4. *Characteristics of THP uptake by M5076 cells*

The effects of extracellular Na^+ and substrate-inhibitors for various transporters on THP uptake by M5076 cells were examined (Fig. 7). The uptake of THP by M5076 cells in the absence of extracellular Na^+ was significantly less than that in the presence of the ion. The uptake of THP by M5076 cells in the presence of extracellular Na^+ was significantly inhibited by uridine, but not by cytidine, to that under the Na^+ free-condition. Furthermore, cholate and taurocholate significantly increased the uptake of THP by M5076 cells, and this enhancement of THP uptake was inhibited by cotreatment with uridine. Other compounds used in this study had no effect on the THP uptake by M5076 cells.

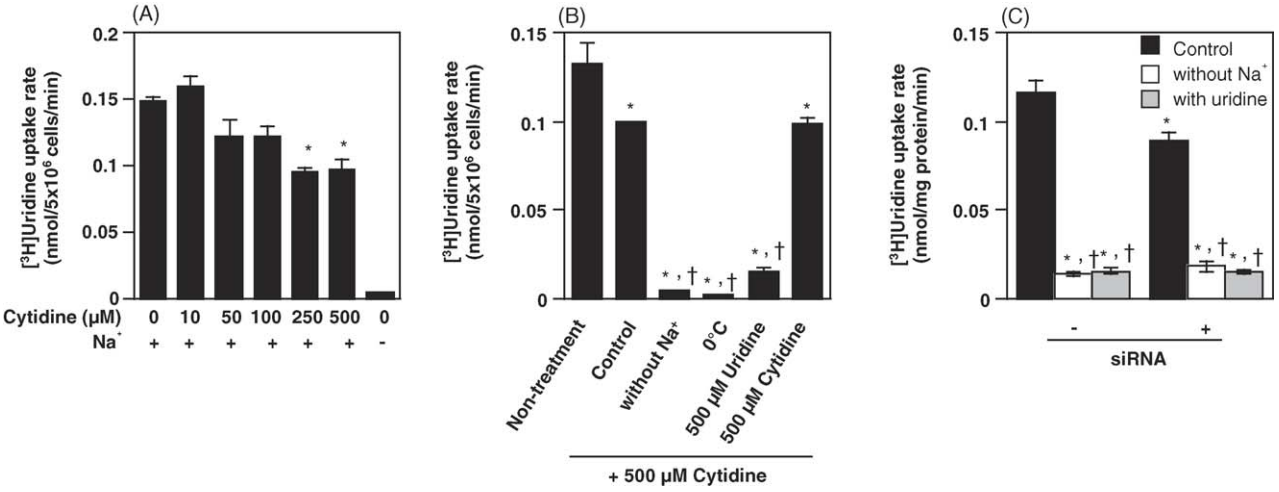

Fig. 4 – Characteristics of [^{3}H]uridine uptake by M5076 cells. (A) The cells were incubated with 5 μM [^{3}H]uridine in HBSS or choline-replaced HBSS containing 10 μM NBMPR and the indicated concentrations of cytidine for 1 min at 37 °C. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, significantly different from group with extracellular Na^+ in the absence of cytidine. (B) The cells were incubated with 5 μM [^{3}H]uridine for 1 min under the designated conditions. The non-treatment group showed the uptake of [^{3}H]uridine without 500 μM cytidine in the presence of extracellular Na^+ at 37 °C. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, $^\dagger p < 0.05$, significantly different from non-treatment and control groups, respectively. (C) The cells were incubated with 5 μM [^{3}H]uridine in HBSS or choline-replaced HBSS containing 10 μM NBMPR in the presence or absence of 500 μM uridine for 1 min at 37 °C. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, $^\dagger p < 0.05$, significantly different from closed bar group in the absence and presence of transfection of siRNA, respectively.

3.5. *Effects of bile acids on the cytotoxicity of THP and DOX toward M5076 cells*

The effects of bile acids on the cytotoxicity of THP and DOX toward M5076 cells were evaluated (Fig. 8), because we previously reported that they were taken up into the cells via the same transporter. The cytotoxicity of THP and DOX toward M5076 cells was significantly enhanced by the cotreatment with both cholate and taurocholate, although these bile acids had no effect on the viability of the cells.

4. Discussion

The present study was performed to clarify whether or not an unidentified transport system for nucleosides exists in M5076

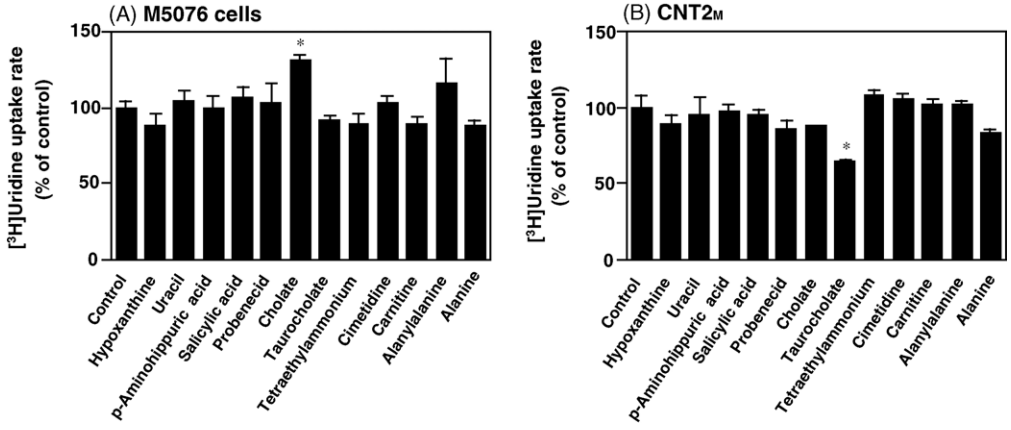

Fig. 5 – Effects of substrate-inhibitors for various transporters on [^{3}H]uridine uptake by M5076 cells and $CNT2_M$. (A) M5076 cells were incubated with 5 μM [^{3}H]uridine in HBSS containing 10 μM NBMPR and the indicated compounds (1 mM, except for alanine (5 mM)) for 1 min at 37 °C. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, significantly different from control. (B) Cos-7/$CNT2_M$ and mock cells were incubated with 5 μM [^{3}H]uridine in HBSS containing 10 μM NBMPR and the indicated compounds (1 mM, except for alanine (5 mM)) for 15 min at 37 °C. The net uptake via $CNT2_M$ was calculated by subtracting the uptake by mock cells from that by Cos-7/$CNT2_M$. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, significantly different from control.

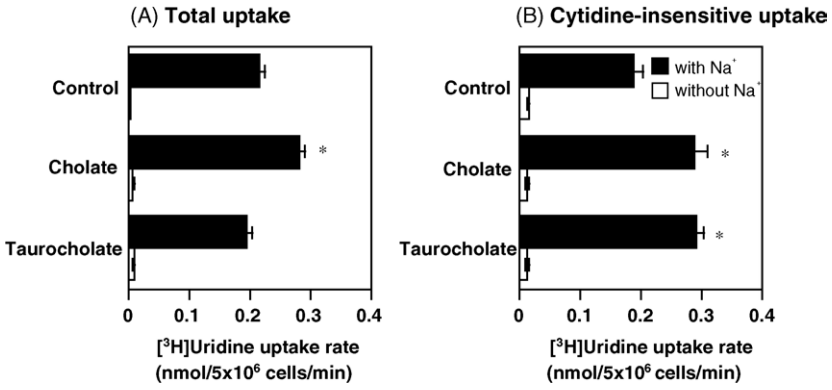

Fig. 6 – Effects of bile acids on [^{3}H]uridine uptake by M5076 cells. The cells were incubated with 5 μM [^{3}H]uridine in HBSS or choline-replaced HBSS containing 10 μM NBMPR and 1 mM of the indicated bile acids in the absence or presence of 500 μM cytidine for 1 min at 37 °C. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, significantly different from each control.

cells, and whether or not anthracyclines are taken up by the system, on the basis of their transport characteristics in the $CNT2_M$ specific siRNA-transfected M5076 cells and the $CNT2_M$-overexpressing Cos-7 cells.

The Na^+-dependent uptake of [^{3}H]uridine by M5076 cells decreased on the addition of 500 μM cytidine only, but significantly to 70% of the control level, although 500 μM cytidine almost completely inhibited the Na^+-dependent uptake of [^{3}H]uridine via $CNT2_M$ and that of [^{3}H]cytidine by M5076 cells. The Na^+-dependent and cytidine-insensitive uptake of [^{3}H]uridine by M5076 cells depended on temperature, and was inhibited by 500 μM uridine, suggesting contribution of a transport system to the uptake. With the M5076 cells transfected with the siRNA specific to $CNT2_M$, the Na^+-dependent [^{3}H]uridine uptake by the cells was reduced only by 23%, although the expression of $CNT2_M$ mRNA was completely disappeared. Therefore, it was indicated that a Na^+-dependent and cytidine-insensitive transport system for nucleosides other than $CNT2_M$ exists in M5076 cells.

Generally, a nucleoside consists of a nucleobase and ribose, and thus it is hypothesized that THP might interact with a nucleoside via the nucleobase, ribose or pentose transporter. Previously, we reported that hexose and pentose transporters were not involved in the Na^+-dependent uptake of uridine and THP by M5076 cells [8], but the contribution of a nucleobase transporter to the uptake remains unclear. Furthermore, nucleoside analogues were reported to be taken up by organic ion transporters and peptide transporters, in addition to NTs

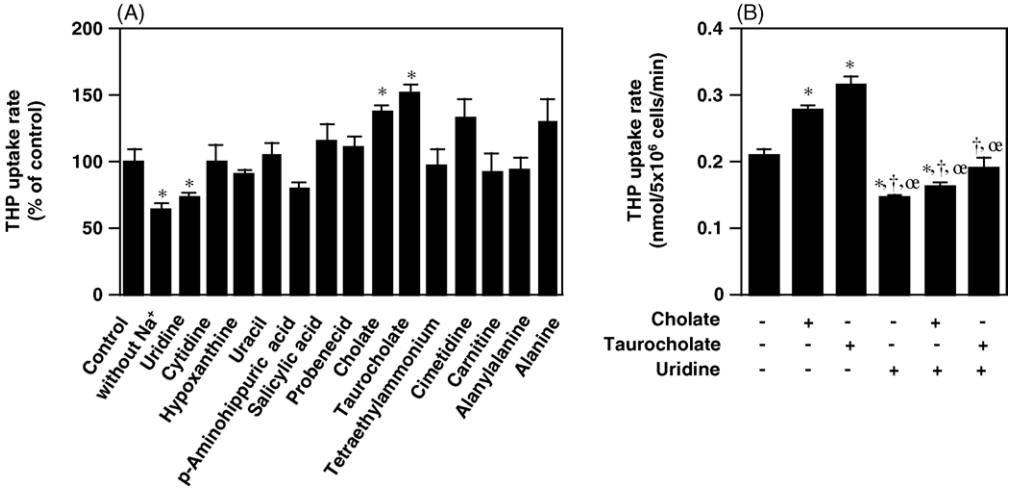

Fig. 7 – Characteristics of THP uptake by M5076 cells. (A) The cells were incubated with 1 μM THP in sodium or choline buffer containing 10 mM AZ, 10 mM DOG, 10 μM NBMPR and the indicated compounds (1 mM, except for nucleoside (500 μM) and alanine (5 mM)) for 1 min at 37 °C. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, significantly different from control. (B) The cells were incubated with 1 μM THP in sodium buffer for 1 min under the designated conditions at 37 °C. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, significantly different from group without bile acids and uridine. $^\dagger p < 0.05$, $^{\text{œ}}p < 0.05$, significantly different from group without uridine in the presence of cholate and taurocholate, respectively.

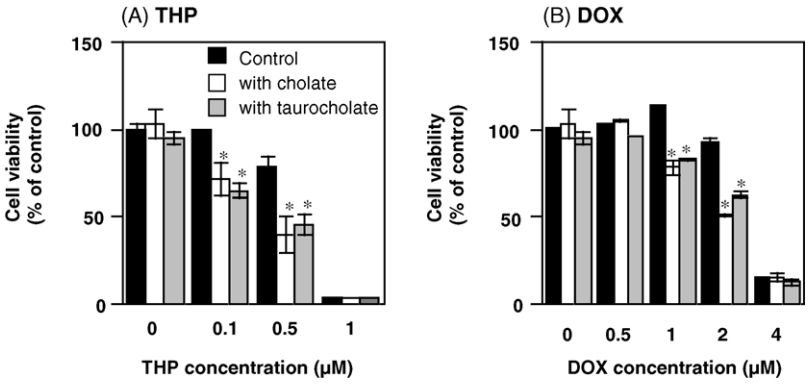

Fig. 8 – Effects of bile acids on cytotoxicity of THP and DOX in M5076 cells. After cells (1×10^6 cells/mL) had been treated with the indicated concentrations of THP and DOX in the presence or absence of cholate or taurocholate for 3 days, MTT assay was performed to evaluate their cytotoxicity. Each bar represents the mean ± S.E. for three experiments. $^*p < 0.05$, significantly different from group without a bile acid.

[18]. Accordingly, we examined effects of substrate-inhibitors for these transporters on [^{3}H]uridine uptake by M5076 cells. As a result of the inhibition assay, the [^{3}H]uridine uptake by M5076 cells was not inhibited by any of the substrate-inhibitors, indicating that the uptake by M5076 cells is not mediated by these transporters. Moreover, although NBMPR-sensitive CNT4 and CNT5 were kinetically identified, NBMPR had no effect on the Na^+-dependent uptake of [^{3}H]uridine by M5076 cells. Therefore, it was thought that the Na^+-dependent and cytidine-insensitive transporter expressed by M5076 cells had not been identified yet. Unexpectedly and interestingly, the uptake of [^{3}H]uridine by M5076 cells with extracellular Na^+ and 500 μM cytidine was increased by both cholate and taurocholate. Furthermore, the effects of the bile acids on [^{3}H]uridine uptake were not observed in the case of $CNT2_M$-transfectants. Thus, it was suggested that these bile acids can enhance the Na^+-dependent uptake of [^{3}H]uridine by M5076 cells, probably by mediating the unidentified transporter.

Next, we examined whether or not the Na^+-dependent and cytidine-insensitive nucleoside transporter is also involved in the uptake of THP by M5076 cells. The uptake of THP by M5076 cells required extracellular Na^+, and was sensitive to uridine, but not cytidine. Furthermore, the uptake of THP by M5076 cells significantly increased on the addition of cholate or taurocholate, as in the case of [^{3}H]uridine uptake by the cells, and the enhancement of THP uptake was reversed by cotreatment with uridine. Therefore, it was indicated that the Na^+-dependent and cytidine-insensitive nucleoside transporter is responsible for the uptake of THP by M5076 cells.

The resistance of tumor cells to anthracyclines is a clinical problem that restricts their usefulness, and one of the major mechanisms underlying the resistance is low expression of topoisomerase II [4]. On the other hand, novel mechanisms for the cytotoxic effects of anthracyclines have been reported, such as induction of apotosis through the generation of hydrogen peroxide [19], and the effects of anthracycline are considered to be enhanced by an increase in their intracellular accumulation, because we have already demonstrated that the uptake efficacy of THP and DOX is responsible for their sensitivities to low topoisomerase II expression M5076 cells [6]. In the present study, the cytotoxicity of THP and DOX toward M5076 cells was enhanced by the cotreatment with both cholate and taurocholate, which was probably mediated by the unidentified Na^+-dependent and cytidine-insensitive nucleoside transporter. Therefore, it is thought that the development of an activator of the unidentified nucleoside transporter will enhance the efficacy of cancer chemotherapy with anthracyclines.

Another interesting observation in our experiment is found in the transport characteristics of $CNT2_M$. [^{3}H]Cytidine uptake by Cos-7/$CNT2_M$ was much greater than that by mock cells, and the uptake via $CNT2_M$ depended on extracellular Na^+ and the substrate concentration. Furthermore, transfection of the $CNT2_M$-specific siRNA into M5076 cells resulted in a great reduction in the Na^+-dependent [^{3}H]cytidine uptake by the cells. To our knowledge, thus, this is the first report demonstrating CNT2-mediated uptake of cytidine. Although we cannot clearly say why the uptake of cytidine via CNT2 has not been observed so far, it might be due to the different expression systems (Cos-7 cells versus Xenopus oocytes). Since NT was recently reported to be a metabolism-driven transporter [20], the great activity of intracellular metabolism of transfectants might be essential to drive the CNT2-mediated uptake of cytidine, comparing with the uridine uptake. On the other hand, thymidine did not act as a substrate for $CNT2_M$, suggesting that the 5-methyl group in the base may prevent the nucleosides from interacting with $CNT2_M$. The K_m value for [^{3}H]cytidine uptake mediated by $CNT2_M$ was significantly greater than the K_i value of cytidine for uptake of [^{3}H]uridine via the transporter, implying that the recognition sites of $CNT2_M$ for cytidine and uridine are possibly different.

Various structural analogs of cytidine are cytotoxic and have found expanding therapeutic uses as antineoplastic agents [10]. In the human uterus, ovary, prostate and lung, the expression of CNT2 has been reported to increase in tumor cells comparing with in the respective normal ones [21]. Furthermore, the great differences among 50 cancer cell lines were seen in the CNT2 genes [22]. Therefore, it was thought that CNT2 is one of the factors dominating the cytotoxic effect

of cytidine analogs, and thus the development of antitumor drugs on the basis of their transport characteristics may lead to enhancement of chemotherapeutic efficacy.

Recently, mechanisms for regulating the transport activity and expression of CNT2 have been vigorously investigated [23–25]. In the present study, [^{3}H]uridine uptake mediated by $CNT2_M$ was significantly decreased by taurocholate, and thus CNT2 may be regulated by multiple substances. On the other hand, the peptide encoded by $CNT2_M$ differed from that encoded by previously cloned mouse CNT2 in five substitutions, but the physiological and pharmacological relationships of the substitutions are unclear. Attempts to determine which transporter is a normal CNT2 one and whether or not transport characteristics differ between them are currently underway.

In conclusion, the present study demonstrated that an unidentified system for the Na^+-dependent transport of nucleosides exists in M5076 cells, in addition to $CNT2_M$, and contributes to the uptake and cytotoxicity of the anthracyclines.

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