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ORIGINAL ARTICLE

Katsuhito Nagai · Kazuki Nagasawa · Sadaki Fujimoto

Uptake of the anthracycline pirarubicin into mouse M5076 ovarian sarcoma cells via a sodium-dependent nucleoside transport system

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Abstract *Purpose*: We have previously demonstrated that the cytotoxicity of anthracyclines, pirarubicin (THP) and doxorubicin (DOX), is partially dominated by their intracellular amounts, which depend on the uptake efficacy of transporter(s). To clarify their transport mechanism, we examined whether or not Na^+/nucleoside cotransporter (CNT) is involved in the uptake of THP by M5076 cells. *Methods*: Expression of the CNT isoforms was determined by reverse-transcription PCR. We used two cell lines, intact M5076 and CNT2-transfected Cos-7 cells, to characterize the uptake of THP and [^{3}H]uridine. *Results*: The mRNA for CNT2, but not that for CNT1 or CNT3, was expressed in M5076 cells, and [^{3}H]uridine uptake by the cells required a Na^+ gradient as a driving force. THP uptake by M5076 cells depended on a Na^+ gradient, and furthermore, formycin B and AZT had *cis*-inhibitory and *trans*-stimulatory effects on the uptake. The efflux of [^{3}H]uridine from M5076 cells was stimulated by the addition of THP extracellularly, which constituted definite evidence of CNT-mediated uptake of THP. However, THP uptake by CNT2 transfectant was almost the same as that by mock cells, indicating that an unidentified CNT isoform contributes to THP uptake by M5076 cells, this being supported by the differences in transport characteristics of [^{3}H]uridine between M5076 and CNT2-transfected cells. *Conclusion*: THP is partially taken up into M5076 cells via a novel Na^+-dependent transport system common to nucleosides.

Keywords Anthracycline · M5076 cell · Nucleoside transport system · De novo resistance · Chemotherapy

K. Nagai (✉) · K. Nagasawa · S. Fujimoto
Department of Environmental Biochemistry,
Kyoto Pharmaceutical University, 5 Nakauchi-cho,
Misasagi, Yamashina-ku, Kyoto 607-8414, Japan
E-mail: nagai@mb.kyoto-phu.ac.jp
Tel.: +81-75-5954648
Fax: +81-755954756

Introduction

Anthracycline antibiotics are some of the most important drugs used for the treatment of a wide variety of human neoplasms, including acute leukemias, lymphomas, stomach, breast and ovarian cancers, Kaposi's sarcomas, and bone tumors [3, 27, 28]. On the other hand, de novo and acquired resistance to these drugs are major clinical problems that restrict their clinical usefulness [17]. Although the mechanism underlying acquired resistance is well clarified [9, 16, 29], there are few findings regarding de novo resistance [14]. Previously, we have demonstrated that the de novo resistance of mouse M5076 ovarian sarcoma cells to doxorubicin (DOX; Fig. 1) is due to an extremely low expression level of topoisomerase II, its target enzyme [25], but that these cells are sensitive to pirarubicin (THP; Fig. 1), the intracellular accumulation of which is greater than that of DOX [19]. The difference in intracellular accumulation between the two drugs is caused by a difference in uptake efficiency of transporter(s), which are responsible for their uptake but not efflux. However, the kind of transport system that is involved in THP uptake by M5076 cells remains unclear.

Nucleoside transporters (NT) play a key role in the physiology of nucleosides and the pharmacology of their analogs in mammals [8, 13, 15]. Mammalian cells possess multiple NTs that are either equilibrative or concentrative, of which five isoforms have been molecularly identified [6, 26]. The equilibrative NTs (ENT), which include transporters sensitive and insensitive to nitrobenzylmercaptopurine (NBMPR) (ENT1 and ENT2, respectively), exhibit broad permeant selectivity [15]. The concentrative NTs (CNT) require an inwardly directed Na^+ gradient as a driving force and are classified into several isoforms based on their substrate specificity [6, 26]. CNT1 and CNT2 transport both uridine and adenosine, but are selective for pyrimidine and purine nucleosides, respectively, and CNT3 shows broad

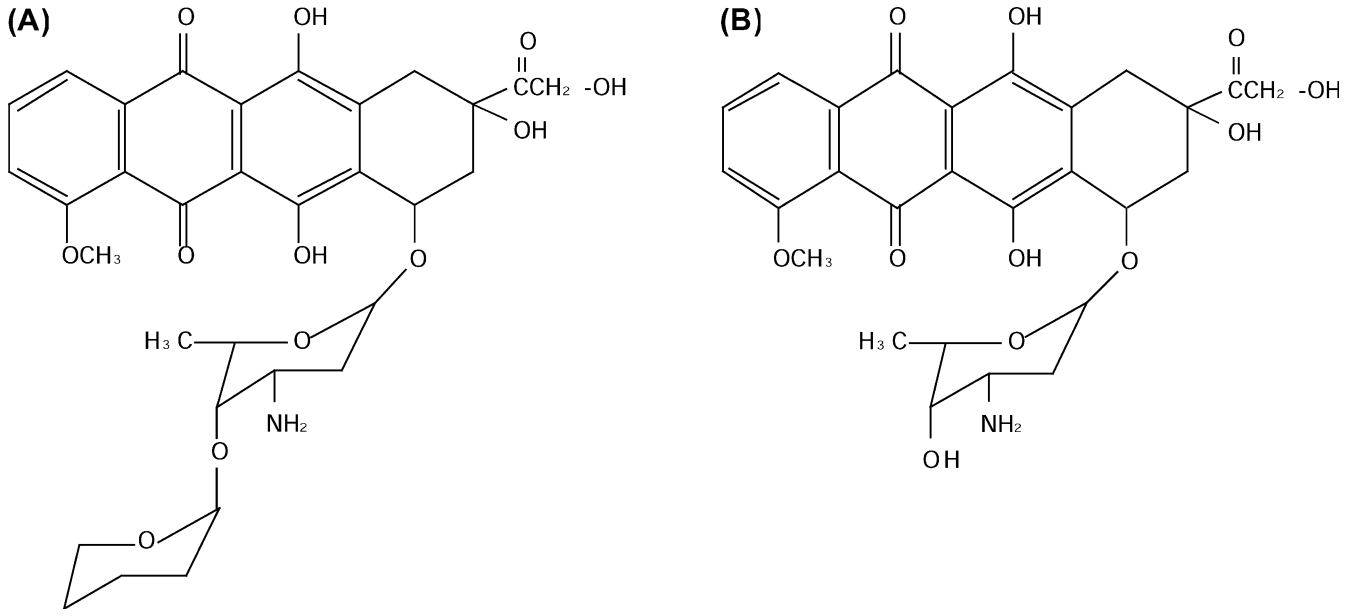


Fig. 1 Chemical structures of (**A**) THP and (**B**) DOX

selectivity or is non-selective. We have been investigating the mechanisms underlying anthracycline transport in tumor cells, and have found that NT may be involved in the uptake of THP and DOX by HL60 cells [22, 23]. Recently, we have found that Na^+-dependent uridine-transportable NT appears to be responsible for the uptake of THP, but not that of DOX, by mouse Ehrlich ascites carcinoma cells [20]. Therefore, it was hypothesized that a CNT isoform(s) may be responsible for THP uptake and that its activity is one of the determinants of the cytotoxicity of THP in M5076 cells.

In this study, therefore, we sought to determine whether or not a CNT(s) was involved in THP uptake by M5076 cells, and characterized the transport of THP and [^{3}H]uridine, a representative substrate for NT, in M5076 cells, in comparison with the CNT isoforms already cloned.

Materials and methods

Chemicals

Pure THP and tetrahydropyranyldoxorubicinol (internal standard for the HPLC assay) were gifts from Meiji Seika Kaisha (Tokyo, Japan). NBMPR, formycin B and 2-deoxy-D-glucose (DOG) were purchased from Sigma Chemical Company (St Louis, Mo.), and uridine, adenosine, inosine, thymidine, cytosine arabinoside (Ara-C), azide thymidine (AZT), and sodium azide (AZ) were from Wako Pure Chemical Industries (Osaka, Japan). [^{3}H]Uridine was obtained from American Radiolabeled Chemicals (St Louis, Mo.). All other reagents were of commercial or analytical grade requiring no further purification.

Cell culture

After M5076 cells [20] had been grown in the abdominal cavity of C57/bl6 male mice (Japan SLC, Hamamatsu, Japan) aged 5–7 weeks, they were isolated and purified by washing with an appropriate buffer. Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (MEM) (Nissui Pharmaceutical Company, Tokyo, Japan) supplemented with 10% fetal bovine serum (ICN Biochemicals, Aurora, Ohio) at 37°C under a humidified atmosphere of 5% CO_2 in air. The density and viability (>90%) were determined by the trypan blue exclusion test.

Reverse transcription (RT) polymerase chain reaction (PCR)

Total RNA was extracted with Sepasol RNA-I super (Nacalai, Kyoto, Japan), and then purified with a GenElute mammalian total RNA kit (Sigma) according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA by means of Oligo-T priming and Moloney murine leukemia virus reverse transcriptase, and then 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). CNT1 was amplified with the 5′ primer 5′-CTGCCATCTCAGT-GAAGG-3′ and the 3′ primer 5′-TCGACAGCAGT-TGTCCAG-3′ to produce a 668 bp product. Because the sequence of murine CNT1 has not been reported, the 5′-primers and 3′-primers were designed on the basis of the sequence conserved in human and rat CNT1 (accession numbers U62966 and U10279, respectively). CNT2 (accession number AF079853) was amplified with the 5′ primer 5′-TTTGGTGATACACT-GGTCC-3′ and the 3′ primer 5′-CCTGACCACAATC-TTGCAC-3′ to produce a 957 bp (bases 748–1704) product. CNT3

(accession number AF305211) was amplified with the 5′ primer 5′-TCTTTGGGGAAAAGTA-TACAG-3′ and the 3′ primer 5′-TCTCATGGCTCCAGAGGCG-3′ to produce a 959 bp (bases 869–1827) product. The PCR product obtained was subcloned into the pGEM-T vector (Promega, UK) and then sequenced.

Cloning of CNT2 cDNA

A CNT2 clone was obtained by amplification of cDNA derived from M5076 cells, using the PCR approach with exTaq (Takara). The 5′ primer 5′-TGAATTCCCAC-CATGGAGAAGTCAAAGGGAAG-3′ and the 3′ primer 5′-TGCGGCCGCTCATGCACACACAGTGCT-GG-3′ derived from the reported sequence of CNT2 in murine liver (accession number AF079853) were used. The PCR product was subcloned into the pGEM-T vector and then sequenced. The CNT2 cDNA was then inserted into the pCI-neo expression vector (Promega) with EcoRI and NotI (ToYoBo, Osaka, Japan).

Transfection

For transfection, pCI-neo and pCI-neo/CNT2 plasmids were purified with a Wizard plus SV Miniprep DNA purification system (Promega). These plasmids were introduced into Cos-7 cells. An appropriate quantity of the required plasmid (26.32 ng of DNA/cm^2) was diluted with 100 μl OPTI-MEM reduced serum medium containing 2.37 nmol/cm^2 of COATSOME EL-01-D (NOF Company, Tokyo, Japan), followed by incubation at room temperature for 30 min to obtain DNA-liposome complexes. The DNA-liposome complexes were added to Cos-7 cells cultured up to 80% confluency, and after 48 h culture, the cells were used for the uptake assay.

Uptake assay

The uptake experiments on M5076 cells were performed by the method of Nagasawa et al. [21] with modification. After M5076 cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG in an appropriate glucose-free medium for 20 min to decrease cellular ATP, the reaction was initiated by adding the indicated concentrations of THP and uridine plus 1.5 μCi ^{3}H-labeled uridine to the pretreated cell suspension (final cell density 5×10^6 cells/ml). For blocking of the function of ENT, the cells were pretreated with metabolic inhibitors and 10 μ*M* NBMPR in choline buffer for 20 min. After appropriate time intervals, the reaction was terminated by the addition of the reaction medium to choline buffer layered over a cushion of silicone oil (sp.gr. 1.050)/mineral oil (sp.gr. 0.845–0.905) (21:4 v/v) in a 1.5-ml microtube, followed by centrifugation for 1 min at 14,000 rpm. In the case of [^{3}H]uridine uptake, to terminate the reaction completely, 1 m*M* unlabeled uridine was added to choline buffer layered over a cushion of oil.

Assaying of the uptake by the transfectants was performed by the method of Nagasawa et al. [24] with modification. The uptake reaction was initiated by adding the indicated concentrations of THP and uridine plus 0.67 μCi ^{3}H-labeled uridine to cells pretreated with 10 μ*M* NBMPR to completely block the NBMPR-sensitive uridine uptake by Cos-7 cells. After appropriate time intervals, the reaction was terminated by adding an excess volume of ice-cold choline buffer containing 1 m*M* unlabeled uridine and 10 μ*M* NBMPR.

The intracellular concentrations of THP and [^{3}H]uridine were determined by HPLC and liquid scintillation counting, respectively, following the methods of Nagasawa et al. [21]. Protein concentrations were determined by the method of Bradford with bovine serum albumin (Sigma) as the standard [4].

Statistical analysis

The data are expressed as means ± SE. The differences between the means of two or more groups were compared using Student's unpaired *t*-test or analysis of variance (ANOVA, followed by Fischer's PLSD), respectively. Differences with a *P* value of 0.05 or less were considered statistically significant.

Results

Characteristics of [^{3}H]uridine uptake by M5076 cells

The time course of [^{3}H]uridine uptake by ENT-blocked M5076 cells in the presence or absence of an inwardly directed Na^+ gradient was examined (Fig. 2A). The uptake of [^{3}H]uridine by M5076 cells in the presence of a Na^+ gradient was much greater than that in the absence of a gradient, and showed the overshoot phenomenon. As shown in Fig. 2B, the rate of uptake of [^{3}H]uridine was significantly reduced by formycin B, a substrate for CNT2 and CNT3, in a dose-dependent manner. [^{3}H]Uridine uptake was also significantly inhibited by thymidine, which has been reported to be a ligand of CNT1 and CNT3, in a dose-dependent manner (Fig. 2C), although the maximum inhibition ranged up to 60.5% of the control level. These results indicate that a CNT(s) is expressed as a functional transporter(s) in M5076 cells.

Expression of mRNA for CNT isoforms in M5076 cells

To check the expression of CNT isoforms in M5076 cells, RT-PCR analysis was performed. As shown in Fig. 3, the message for CNT was detected in mouse kidney (CNT1) or intestine (CNT2 and CNT3) as a positive control, the primer sets used being confirmed to

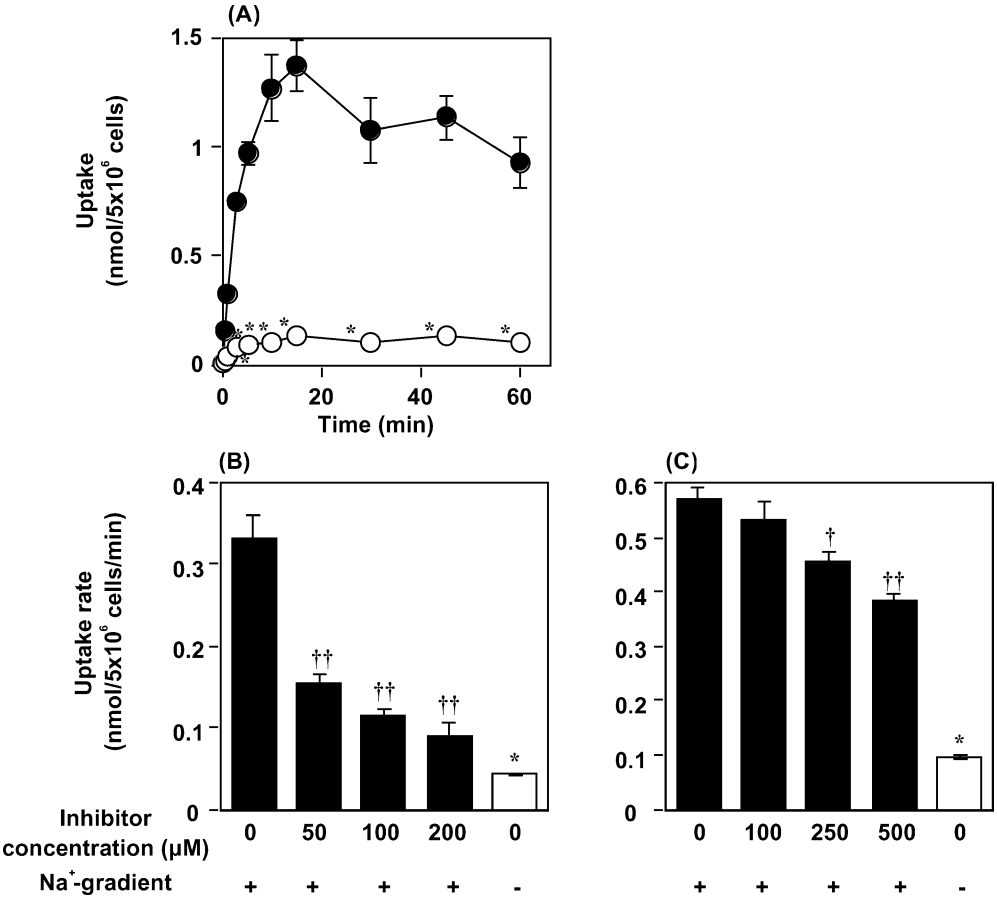


Fig. 2 Effects of (**A**) an inwardly directed Na^+ gradient, (**B**) formycin B, and (**C**) thymidine on [^{3}H]uridine uptake by ENT-blocked M5076 cells. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 20 min in choline buffer, they were incubated with 5 μ*M* [^{3}H]uridine in the presence or absence of the indicated concentrations of formycin B or thymidine in sodium (*closed*) or choline (*open*) buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for the indicated times at 37°C. The presented data are the means ± SE from three experiments. $^*P < 0.01$ vs uptake in the presence of a Na^+ gradient; $^\dagger P < 0.05$ and $^{\dagger\dagger} P < 0.01$ vs uptake without formycin B or thymidine in the presence of a Na^+ gradient, respectively

be valid. In M5076 cells, one PCR product of the expected mobility for CNT2 was observed, i.e., not that for CNT1 and CNT3. Furthermore, the results were similar for the two different primer sets specific to each CNT (data not shown). Therefore, CNT2, but not CNT1 and CNT3, was expressed in M5076 cells, without their alternative splicing.

Characteristics of THP uptake by M5076 cells

The effects of an inwardly directed Na^+ gradient and CNT inhibitors on THP uptake by M5076 cells are shown in Figs. 4 and 5. The uptake of THP by M5076 cells in the absence of a Na^+ gradient was significantly less than that in the presence of a gradient (Fig. 4A). All of the CNT inhibitors used apparently decreased the rate of uptake of THP by M5076 cells in the presence, but not in the absence, of an inwardly directed Na^+ gradient (Fig. 4B).

The *cis*-inhibitory and *trans*-stimulatory effects of formycin B and AZT on the THP uptake by M5076 cells were determined to elucidate the mechanism underlying their interaction (Fig. 5). THP uptake by the cells was competitively inhibited by formycin B and AZT with apparent inhibition constants (Ki) of 256.9 and 288.5 μ*M*, respectively (Fig. 5A,B). As shown in Fig. 5C,D, the uptake of THP significantly increased with increasing preloaded amounts of formycin B or AZT.

Interaction of [^{3}H]uridine and THP uptake

Na^+-dependent uptake of [^{3}H]uridine by M5076 cells was significantly inhibited by THP in a dose-dependent manner (Fig. 6A). Figure 6B shows the effect of THP on the efflux of [^{3}H]uridine from M5076 cells. The efflux of [^{3}H]uridine from the cells in the presence of an outwardly directed Na^+ gradient was significantly accelerated by the addition of 25 μ*M* THP extracellularly. Thus, THP and uridine have been shown to be taken up Na^+-dependently via the same transporter in M5076 cells.

[^{3}H]Uridine and THP uptake by CNT2-transfected cells

The uptake of [^{3}H]uridine by ENT-blocked Cos-7 cells transfected with pCI-neo (mock), as a control, and pCI-neo/CNT2 (Cos-7/CNT2) was evaluated to confirm the functional expression of the transfected CNT2 in Cos-7 cells. [^{3}H]Uridine uptake over a 30-min period was

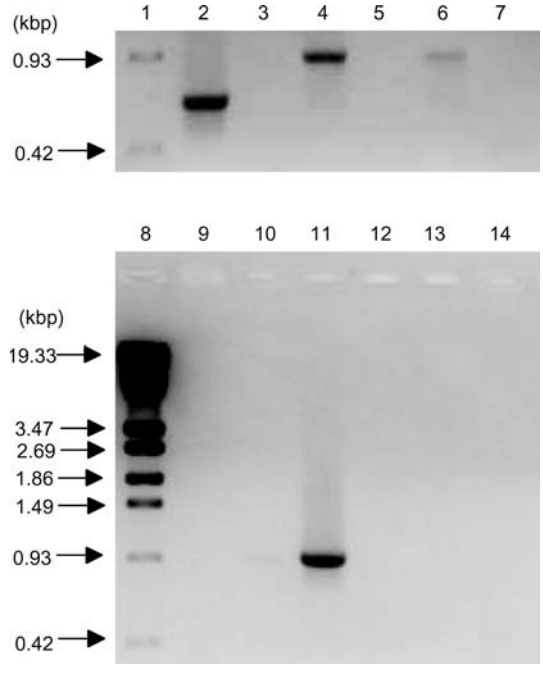


Fig. 3 Expression of mRNA for CNTs in M5076 cells on RT-PCR analysis. Total RNA was extracted as described in "Materials and methods", and used for RT-PCR. PCR product was loaded at 12 μl per lane. *Lanes 1 and 8* size markers (*λ*/StyI digest); *lanes 2 and 3* CNT1 in mouse kidney with and without RT, respectively; *lanes 4 and 5* CNT2 in mouse intestine with and without RT, respectively; *lanes 6 and 7* CNT3 in mouse intestine with and without RT, respectively; *lanes 9 and 10* CNT1 in M5076 cells with and without RT, respectively; *lanes 11 and 12* CNT2 in M5076 cells with and without RT, respectively; *lanes 13 and 14* CNT3 in M5076 cells with and without RT, respectively

apparently greater for Cos-7/CNT2 than for mock, indicating that the transfected CNT2 acts as a membrane transporter (Fig. 7A). The uptake of [^{3}H]uridine via the recombinant transporter clearly depended on an inwardly directed Na^+ gradient and was significantly reduced by CNT2 substrates, uridine, adenosine and formycin B, while thymidine, a substrate for CNT1 and CNT3, had no effect on the uptake of [^{3}H]uridine (Fig. 7B).

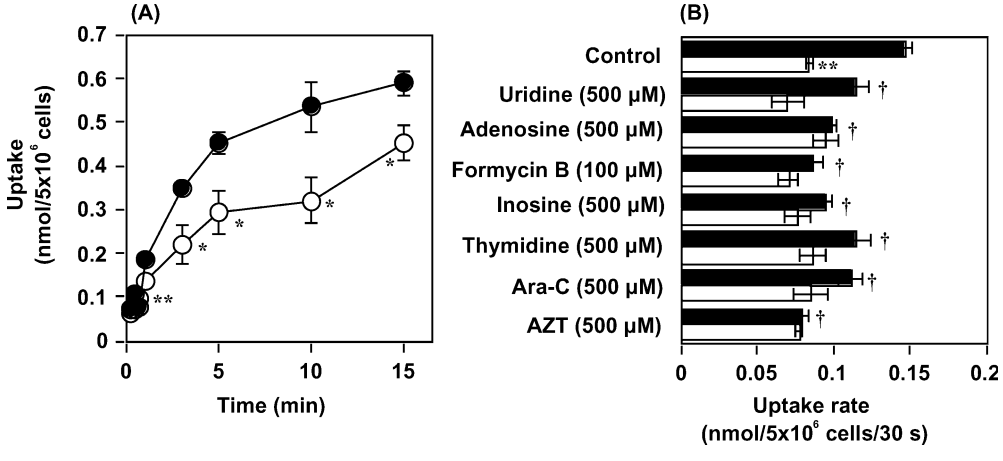
On the other hand, THP had no effect on the uptake of [^{3}H]uridine via CNT2 (Fig. 8A), and the uptake of THP by Cos-7/CNT2 was very similar to that by mock (Fig. 8B). Overall, it appears that the CNT2 cloned from M5076 cells does not transport THP.

Discussion

Firstly, to determine whether or not a CNT is functionally expressed in M5076 cells, [^{3}H]uridine uptake by M5076 cells was characterized. The uptake of [^{3}H]uridine by M5076 cells apparently depended on an inwardly directed Na^+ gradient as a driving force, and was dose-dependently inhibited by various nucleosides. Accordingly, a CNT(s) was confirmed to function as a membrane transporter in M5076 cells. This was supported by the finding on RT-PCR analysis that the mRNA for CNT2, but not that of CNT1 or CNT3, was presented in M5076 cells.

Thus, we investigated the involvement of CNT in the uptake of THP by M5076 cells. The uptake of THP by ENT-blocked M5076 cells depended on an inwardly directed Na^+ gradient, and the Na^+ gradient-dependent uptake was almost completely inhibited by nucleosides and their analogs. Also, THP uptake by ENT-blocked M5076 cells was competitively inhibited by formycin B and AZT, and was stimulated with their preloading into the cells. Thus, the Na^+ gradient-dependent uptake of

Fig. 4 Effects of (**A**) an inwardly directed Na^+ gradient and (**B**) nucleosides on THP uptake by M5076 cells. **A** After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG for 20 min in choline buffer, they were incubated with 1 μ*M* THP in sodium (*closed*) or choline (*open*) buffer containing 10 m*M* AZ plus 10 m*M* DOG for the indicated times at 37°C. **B** After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 20 min in choline buffer, they were incubated with 1 μ*M* THP and the indicated concentrations of a nucleoside in sodium (*closed*) or choline (*open*) buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 30 s at 37°C. The presented data are the means ± SE from three experiments. $^*P < 0.05$ and $^{**}P < 0.01$ vs uptake in the presence of a Na^+ gradient, respectively; $^\dagger P < 0.01$ vs each control group



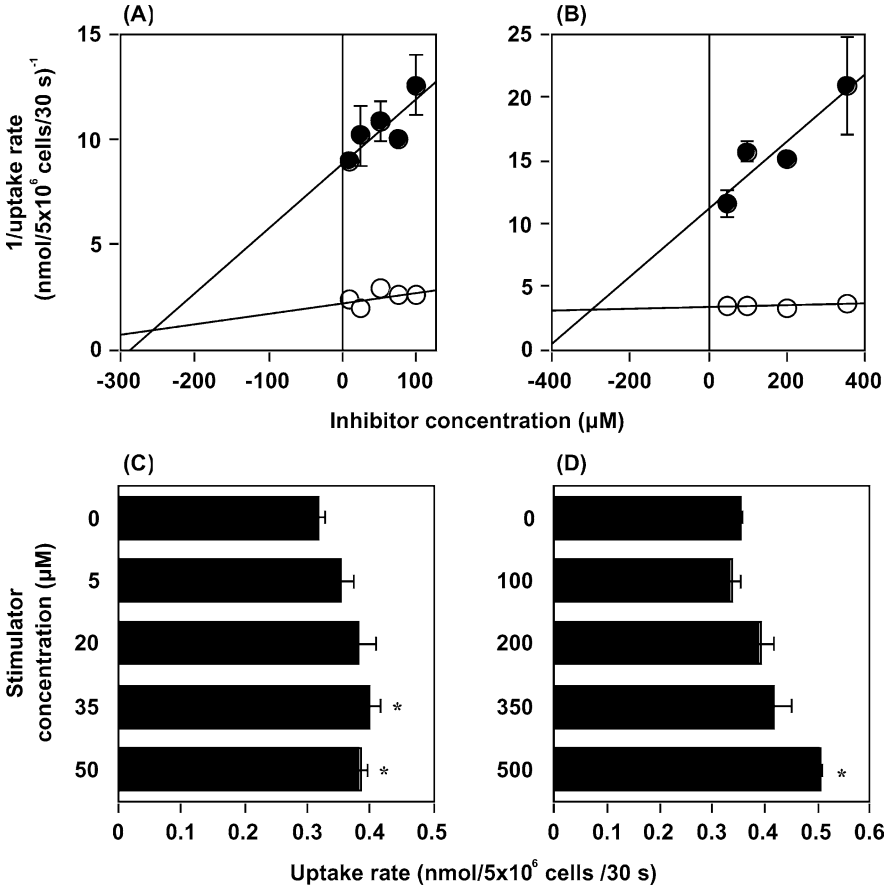


Fig. 5 *Cis*-inhibitory (**A**,**B**) and *trans*-stimulatory (**C**,**D**) effects of formycin B and AZT on THP uptake by ENT-blocked M5076 cells. **A**, **B** After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 20 min in choline buffer, they were incubated with 1 μ*M* (*closed*) or 5 μ*M* (*open*) THP and the indicated concentrations of (**A**) formycin B or (**B**) AZT in sodium buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 30 s at 37°C. The presented data are the means ± SE from three experiments. The Ki values of formycin B and AZT for THP uptake by M5076 cells were calculated to be 256.9 μ*M* and 288.5 μ*M*, respectively. **C**,**D** After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and the indicated concentrations of (**C**) formycin B or (**D**) AZT for 15 min in choline buffer, they were incubated with 5 μ*M* THP in sodium buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 30 s at 37°C. Each bar represents the mean ± SE from three experiments. $*P < 0.05$ vs uptake without a stimulator

THP is mediated by the same system(s) as for these nucleosides.

On the basis of the above findings, we sought to determine whether or not THP is taken up via CNT2 using CNT2-transfected cells. The time course of [^{3}H]uridine uptake was much greater for Cos-7/CNT2 than that for mock. In addition, [^{3}H]uridine uptake via CNT2 depended on an inwardly directed Na^+ gradient and exhibited almost the same substrate specificity as that already reported, indicating that transfection of cDNA for CNT2 into Cos-7 cells resulted in the expression of its protein as a membrane transporter. However, the uptake of THP via CNT2 was not observed in the experiment on this CNT2 transfectant, suggesting that THP is not taken up via CNT2. These findings suggest that a system for the Na^+-dependent transport of nucleosides, besides CNT1, CNT2 and CNT3, is responsible for THP uptake by M5076 cells.

There were some differences in the transport characteristics of [^{3}H]uridine between M5076 and CNT2-transfected Cos-7 cells. Namely, the degree of the inhibitory effect of formycin B on [^{3}H]uridine uptake by M5076 cells was significantly greater than that of the recombinant CNT2. Furthermore, thymidine and THP significantly inhibited the uptake of [^{3}H]uridine by M5076 cells, but CNT2 did not. Because the Na^+-dependent transport of [^{3}H]uridine by M5076 cells could not be fully explained by CNT2, a novel system for Na^+-dependent nucleoside transport was thought to be present in M5076 cells, and this is supported by the uptake characteristics of THP in the cells. Overall, it is suggested that THP was taken up via an unidentified Na^+-dependent NT expressed in M5076 cells. In our laboratory, attempts to clarify whether this system is a new CNT isoform or another transporter involved in the uptake of nucleosides are currently underway.

Confirming that anthracycline is taken up by a specific transporter has previously been believed to be difficult because of the self-association of anthracycline and/or the formation of a complex between anthracycline and nucleoside [1, 2, 10]. However, in this study,

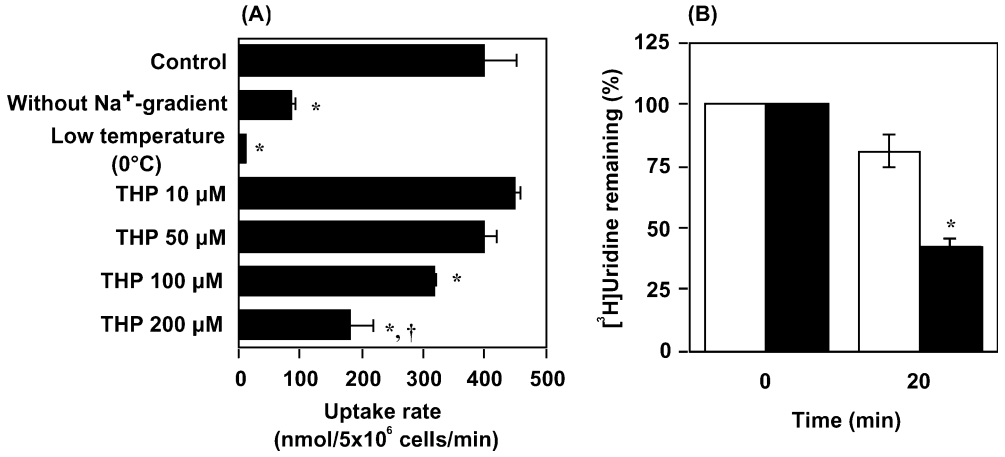


Fig. 6 Effect of THP on [^{3}H]uridine transport in ENT-blocked M5076 cells. **A** After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 µ*M* NBMPR for 20 min in choline buffer, they were incubated with 5 µ*M* [^{3}H]uridine and the indicated concentrations of THP in sodium or choline buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 µ*M* NBMPR for 1 min at 0°C or 37°C. Each bar represents the mean ± SE from three experiments. $^*P < 0.001$ vs control: $^\dagger P < 0.05$ vs uptake without a Na^+ gradient. **B** After cells had been loaded with 5 µ*M* [^{3}H]uridine for 15 min in sodium buffer containing 10 m*M* AZ plus 10 m*M* DOG, they were incubated with (*closed*) or without (*open*) 25 µ*M* THP in choline buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 µ*M* NBMPR for 20 s. Each bar represents the mean ± SE from three experiments. $^*P < 0.05$ vs uptake without preloading of THP

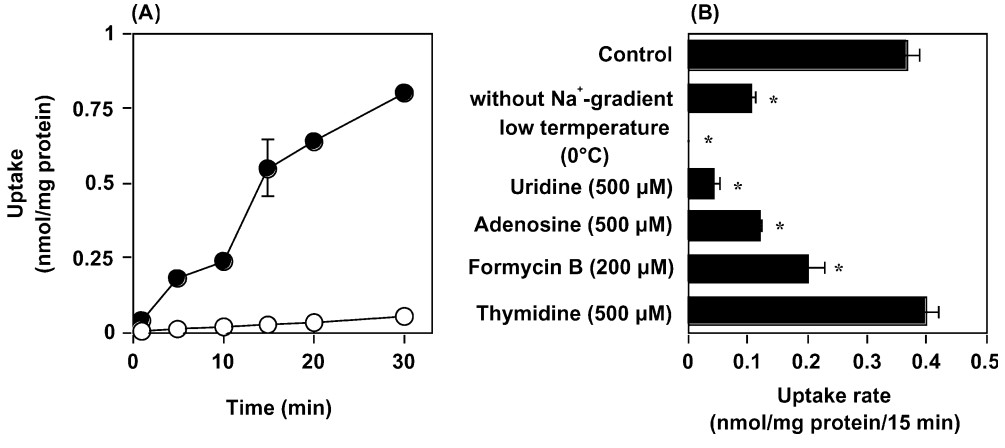


Fig. 7 Characteristics of [^{3}H]uridine uptake via CNT2 expressed in Cos-7 cells. **A** After Cos-7/CNT2 (*closed*) and mock (*open*) had been pretreated with 10 µ*M* NBMPR for 5 min in Hank's balanced salt solution (HBSS), they were incubated with 5 µ*M* [^{3}H]uridine in HBSS containing 10 µ*M* NBMPR for the indicated times at 37°C. Each point represents the mean ± SE from three experiments. **B** After cells had been pretreated with 10 µ*M* NBMPR for 5 min in HBSS or choline-replaced HBSS, they were incubated with 5 µ*M* [^{3}H]uridine in an appropriate buffer containing 10 µ*M* NBMPR in the presence or absence of the indicated concentration of a nucleoside for 15 min at 0°C or 37°C. The net uptake via CNT2 was calculated by subtracting the uptake by mock from that by Cos-7/CNT2. Each bar represents the mean ± SE from three experiments. $^*P < 0.001$ vs control

[^{3}H]uridine uptake by M5076 cells, but not by CNT2-transfected Cos-7 cells, was inhibited by THP in a dose-dependent manner, although the Na^+-dependent [^{3}H]uridine uptake by M5076 cells was not completely inhibited by 200 µ*M* THP. This incomplete inhibition by THP was thought to be due to its concentration as an inhibitor, which is almost identical to the apparent Michaelis constant for THP uptake by M5076 cells (198.5 µ*M*). In addition, [^{3}H]uridine efflux from ENT-blocked M5076 cells was accelerated by adding THP extracellularly. This is considered to result from acceleration of the conformational change of the transporter by extracellular THP, but not from the self-association of THP, since Yao et al. consider that *trans*-acceleration of nucleoside efflux is a powerful methodology for finding nucleoside analogs such as CNT permeants [30]. Therefore, we think that these findings constitute definite kinetic evidence of transporter-mediated uptake of THP by M5076 cells.

The uptake of [^{3}H]uridine by M5076 cells was inhibited by THP in a dose-dependent manner. Nucleosides, including uridine, are known to be essential for cell survival and growth [13]. Tumor cells are thought to require more nucleosides than normal cells, and so expression of the majority of NT isoforms has been reported to be increased in tumor cells compared with their expression in the respective normal cells [12, 18]. Thus, inhibition of nucleoside uptake via a transporter

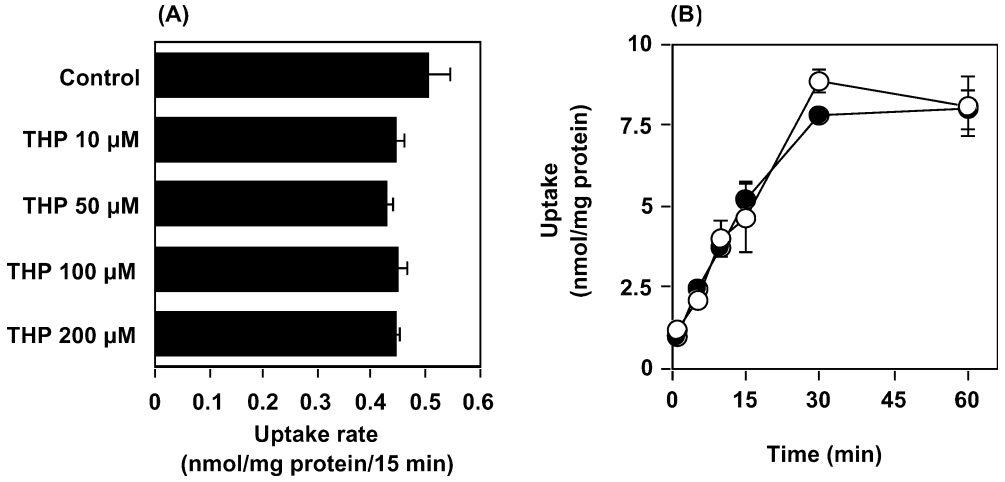


Fig. 8 Effect of THP on [^{3}H]uridine uptake (**A**) and THP uptake via CNT2 (**B**). **A** After cells had been pretreated with 10 μ*M* NBMPR for 5 min in HBSS, they were incubated with 5 μ*M* [^{3}H]uridine in HBSS containing 10 μ*M* NBMPR and the indicated concentrations of THP for 15 min at 37°C. The net uptake via CNT2 was calculated by subtracting the uptake by mock from that by Cos-7/CNT2. Each bar represents the mean ± SE from three experiments. **B** After Cos-7 (*open*) and Cos-7/CNT2 (*closed*) had been pretreated with 10 μ*M* NBMPR for 5 min in HBSS, they were incubated with 10 μ*M* THP in HBSS containing 10 μ*M* NBMPR for the indicated times at 37°C. Each point represents the mean ± SE from three experiments

by THP is a newly discovered mechanism underlying its cytotoxicity. Moreover, it is expected that the combination of an inhibitor of nucleoside transport and an inhibitor of de novo nucleotide biosynthesis would be able to enhance the effectiveness of chemotherapy by modulating either drug influx or drug efflux, and by interfering with the salvage pathways for DNA synthesis [5, 7, 11]. Therefore, the development of anthracyclines on the basis of transport characteristics may lead to enhancement of chemotherapeutic efficacy.

In conclusion, the findings of the present study indicate that a novel system for Na^+-dependent uptake of nucleosides expressed in M5076 cells contributes to THP uptake and provide a new insight into the development of new cancer chemotherapies.

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