Cancer Chemother Pharmacol (2003) 51: 512–518
DOI 10.1007/s00280-003-0569-0

ORIGINAL ARTICLE

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Pirarubicin is taken up by a uridine-transportable sodium-dependent concentrative nucleoside transporter in Ehrlich ascites carcinoma cells

Received: 30 August 2002 / Accepted: 13 December 2002 / Published online: 2 April 2003

Abstract *Purpose*: We evaluated the contribution of a nucleoside transporter (NT) consisting of an equilibrative NT (ENT) and a concentrative Na^+/nucleoside cotransporter (CNT) to the uptake of THP and DOX by mouse Ehrlich ascites carcinoma cells. *Methods*: Transport experiments were performed using a silicone layer method. The expression of CNT isoforms was confirmed by RT-PCR analysis. *Results*: The effects of inhibition of the ENT inhibitors, nitrobenzylthioinosine (NBMPR) and nitrobenzylthioguanosine, on THP and DOX uptake by Ehrlich cells was negligible. THP uptake, but not DOX uptake, partially depended on an inwardly directed Na^+ gradient, and the uptake was inhibited by all the inhibitors of CNT examined. Furthermore, efflux of [^{3}H]uridine from Ehrlich cells was stimulated by the addition of THP to the extracellular compartment, which was definitive evidence of CNT-mediated uptake of THP. The mRNA for CNT2, but not that for CNT3, was detected in Ehrlich cells, which is consistent with the characteristics of [^{3}H]uridine uptake. In the cells, formycin B, a representative CNT2 ligand, had *cis*-inhibitory and *trans*-stimulatory effects on THP uptake. *Conclusion*: These results demonstrate that THP, but not DOX, is taken up into Ehrlich cells partially via a uridine-transportable CNT.

Keywords Anthracycline · Transport · Ehrlich cell · Nucleoside transporter · Molecular target

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Introduction

Anthracyclines are the most active chemotherapeutic agents in a variety of malignancies [3, 28]. These drugs, however, induce dose-dependent leukopenia, cardiac myopathy, alopecia, etc., which restricts their clinical usefulness. These adverse effects occur because of their distribution not only in tumor cells but also in normal cells.

Anthracyclines are believed to be taken up through passive diffusion on the basis of the finding of self-association with high concentrations of anthracyclines [2, 6, 10] resulting in a transporter-like property [7, 8, 9]. On the other hand, we (reference 18 and references therein), Skovsgaard [22, 23, 24, 25], Slapak et al. [26], and Sasaya et al. [21] have found strong evidence that transporters are involved in anthracycline uptake, although the possibility that self-association may have interfered with the experimental results cannot be completely ruled out. Thus, whether or not a transporter contributes to anthracycline uptake is a matter of controversy.

We have been investigating the characteristics of anthracycline transport to establish a strategy for selectively delivering anthracyclines to tumor cells utilizing differences in the membrane transport characteristics between tumor and normal cells [18]. In our previous studies, we have found that pirarubicin (THP) and doxorubicin (DOX) (Fig. 1) are possibly taken up by certain nucleoside transporters (NTs), including equilibrative NT (ENT) and concentrative Na^+/nucleoside cotransporter (CNT), in human leukemia HL60 cells, but not in human mononuclear cells [18]. Furthermore, it has been reported that in some tissues the expression of NT is increased in tumor cells compared with the expression in normal cells [19]. Therefore, NT-mediated transport of antitumor agents including anthracyclines may be a critical strategy.

Recently, we have found that THP and DOX are taken up by Ehrlich ascites carcinoma cells via different

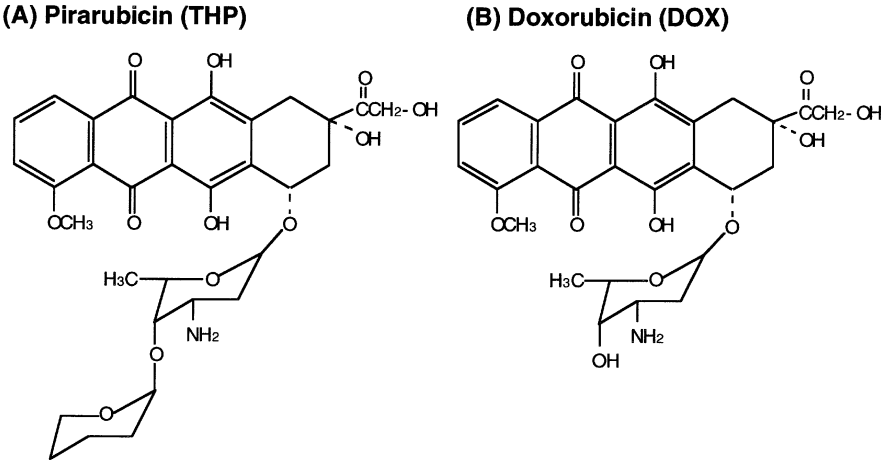

Fig. 1 Chemical structures of pirarubicin and doxorubicin

transport systems [15], but what kinds of transport systems are involved was not established. In this study, therefore, we assessed the contribution of NT to THP and DOX uptake by Ehrlich cells, in which NT is expressed more than in HL60 cells [4, 11, 12, 13, 14], and also identified the NT isoform involved in the uptake by kinetic and mRNA analyses. THP, but not DOX, was found to be taken up by Ehrlich cells via mouse uridine-transportable CNT. To our knowledge, this is the first study in which the transporter-mediated uptake of an anthracycline has been demonstrated.

Material and methods

Chemicals

Pure THP (pirarubicin) and tetrahydropyranyldoxorubicinol (internal standard for the HPLC assay were obtained from Meiji Seika Kaisha (Tokyo) and DOX (doxorubicin hydrochloride) was obtained from Kyowa Hakko Kogyo Company (Tokyo). Nitrobenzylthioinosine (NBMPR), nitrobenzylthioguanosine (NBTGR), formycin B and 2-deoxy-D-glucose (DOG) were purchased from Sigma Chemical Co. (St. Louis, Mo.), and uridine, adenosine, inosine, thymidine, cytosine arabinoside (Ara-C), azidethymidine (AZT) and sodium azide (AZ) from Wako Pure Chemical Industries (Osaka). [^{3}H]Uridine was from American Radiolabeled Chemicals (St. Louis, Mo.). All other reagents were of commercial or analytical grade requiring no further purification. In this study, we used glucose-free Hanks' balanced salt solution (HBSS, pH 7.4) and choline-replaced glucose-free HBSS as sodium and choline buffers, respectively.

Preparation of Ehrlich cells

After Ehrlich cells (kindly provided by Dr. Y. Sadzuka, University of Shizuoka) had been grown in the abdominal cavity of ddY male mice (SLC, Hamamatsu, Japan) aged 5–6 weeks, they were isolated and purified by washing with an appropriate transport buffer (purity > 95%). The density and viability (> 90%) were determined by the trypan blue exclusion test.

Anthracycline uptake experiments

The experiments were performed by the modified method of Nagasawa et al. [16]. After the cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG in an appropriate glucose-free medium for 20 min to deplete cellular ATP, the reaction was initiated by the addition of the indicated concentrations of THP or DOX to the incubation medium including the cells (final density 5×10^6 cells/ml). After appropriate times, the reaction was terminated by the addition of the reaction medium to choline buffer layered over a cushion of silicone oil/mineral oil (specific gravities 1.050 and 0.845–0.905, respectively; 21:4 v/v) in a 1.5-ml microtube, followed by centrifugation for 1 min at 10,000 rpm. The concentration of each nucleoside transport inhibitor used was based upon the findings of our previous study [17] to give approximately the maximum inhibitory effect. The THP and DOX concentrations in Ehrlich cells were determined by HPLC according to the method of Nagasawa et al. [16].

[^{3}H]Uridine uptake experiments

The uptake reaction was initiated by the addition of 5 μ*M* uridine containing 2.5 μCi of the ^{3}H-labeled form to the cell suspension. At the end of the incubation period, the reaction was terminated by the addition of the reaction medium to a 1 m*M* uridine-containing solution layered over a cushion of silicone oil/mineral oil as described above, followed by immediate centrifugation. The cells were solubilized with 1 *N* NaOH, neutralized with 5 *N* HCl, and then transferred to vials for liquid scintillation counting after the addition of Scintizol EX-H (Dojin Chemicals, Kumamoto, Japan).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from Ehrlich cells with a Gen Elute Mammalian Total RNA kit (Sigma Chemical Company, St. Louis, Mo.) according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA with Oligo-T priming and Moloney murine leukemia virus reverse transcriptase, and then the cDNA was PCR-amplified at 94°C for 15 s, 50°C for 30 s and 72°C for 60 s with Ex-Taq DNA polymerase (TaKaRa, Japan). CNT2 (accession number AF079853) was amplified with the 5′ primer 5′-TTTGGTGATACACTGGTCC-3′ and the 3′ primer 5′-CCTGACCACAATCTTGCAC-3′ to produce a 957-bp product (bases 748 to 1704). CNT3 (accession number AF305211) was analyzed with the 5′ primer 5′-TCTTTGGGGAAAAGTATACAG-3′ and the 3′ primer 5′-TCTCATGGCTCCAGAGGCG-3′ to produce a 959-bp product (bases 869 to 1827). Each PCR product obtained was subcloned into the pGEM-T vector (Promega, Southampton, UK) and sequenced.

Statistical analysis

The data are expressed as means ± SE. Comparisons between two or more groups were performed by means of Student's unpaired

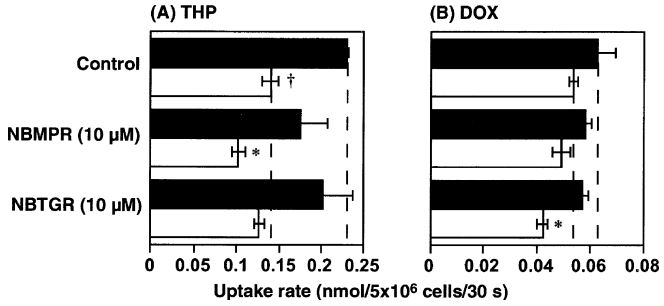

Fig. 2A, B Effects of ENT inhibitors on THP and DOX uptake by Ehrlich cells in the presence or absence of an inwardly directed Na^+ gradient. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG for 20 min in choline buffer, they were incubated with 1 μ*M* THP or DOX and the indicated concentrations of an inhibitor in sodium (*closed bars*) or choline (*open bars*) buffer containing 10 m*M* AZ plus 10 m*M* DOG for 30 s at 37°C. Each bar represents the mean ± SE from three experiments. $^*P < 0.05$ vs each control group, $^\dagger P < 0.05$ vs the control value in the presence of an inwardly directed Na^+ gradient

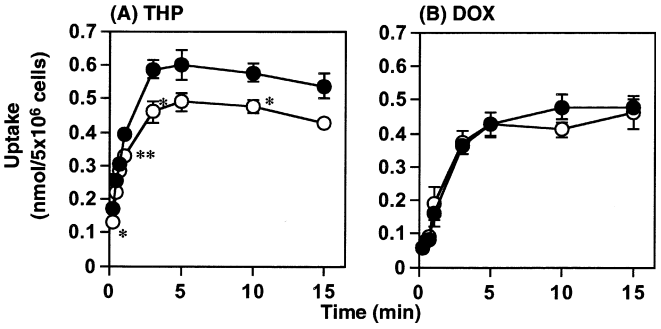

Fig. 3A, B Time courses of THP and DOX uptake by Ehrlich cells in the presence or absence of an inwardly directed Na^+ gradient. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG for 20 min in choline buffer, they were incubated with 1 μ*M* THP or DOX in sodium (●) or choline (○) buffer containing 10 m*M* AZ plus 10 m*M* DOG for the indicated times at 37°C. Each point represents the mean ± SE from three to six experiments. $^*P < 0.05$, $^{**}P < 0.01$, vs the respective value in the presence of an inwardly directed Na^+ gradient at the corresponding time point

t-test or analysis of variance (ANOVA) followed by Fischer's PLSD. Differences with a *P* value of 0.05 or less were considered statistically significant.

Results

Effects of ENT inhibitors on THP and DOX uptake

Figure 2 shows the effects of ENT inhibitors on the uptake of THP and DOX by Ehrlich cells. The rates of uptake of THP and DOX in the absence of an inwardly directed Na^+ gradient decreased with the addition of 10 μ*M* NBMPR or NBTGR, which has been reported to inhibit completely the uptake of uridine and formycin B via ENT in Ehrlich cells [4]. On the other hand, these inhibitors had no effect on THP uptake in the presence of the gradient. The finding that [^{3}H]uridine uptake decreased to 28% on pretreatment of the cells with 10 μ*M* NBMPR (data not shown) indicates that ENT is expressed in Ehrlich cells and that THP and DOX are taken up by Ehrlich cells via ENT, although the contribution is low.

Effects of a Na^+ gradient on THP and DOX uptake

The time courses of THP and DOX uptake by Ehrlich cells in the presence or absence of an inwardly directed Na^+ gradient are presented in Fig. 3. The uptake of THP in the absence of a Na^+ gradient was significantly decreased up to about 80% compared with that in the presence of a Na^+ gradient, while the uptake of DOX in the presence and absence of a Na^+ gradient was approximately the same at each time point. These results indicate that an inwardly directed Na^+ gradient is a driving force for THP uptake by Ehrlich cells, although its contribution is partial. Thus, in the following experiments, only THP uptake was examined.

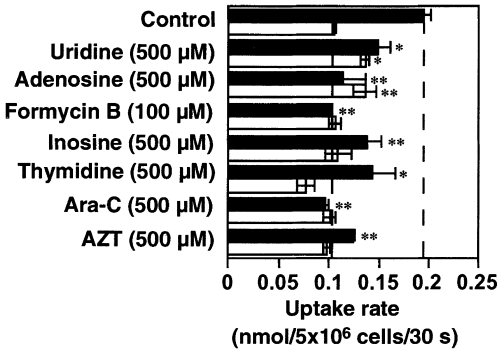

Fig. 4 Effects of CNT inhibitors on THP uptake by ENT-blocked Ehrlich cells in the presence or absence of an inwardly directed Na^+ gradient. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 20 min in choline buffer, they were incubated with 1 μ*M* THP and the indicated concentrations of an inhibitor in sodium (*closed bars*) or choline (*open bars*) 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. $^*P < 0.05$, $^{**}P < 0.01$, vs each control group; $^\dagger P < 0.01$ vs the control value in the presence of an inwardly directed Na^+ gradient

Effects of CNT inhibitors on THP uptake

All the CNT inhibitors used in this experiment significantly decreased the rate of uptake of THP by ENT-blocked Ehrlich cells in the presence of an inwardly directed Na^+ gradient (Fig. 4). Furthermore, the decreased rate of uptake of THP with a Na^+ gradient on treatment with each inhibitor, except for inosine and thymidine, was approximately equal to that without the gradient. To examine the inhibitory mechanism of CNT inhibitors, the *cis*-inhibitory and *trans*-stimulatory effects on THP uptake of formycin B and AZT, by which THP uptake depending on an inwardly directed Na^+ gradient was completely inhibited, were examined (Fig. 5). The uptake of THP was competitively inhibited

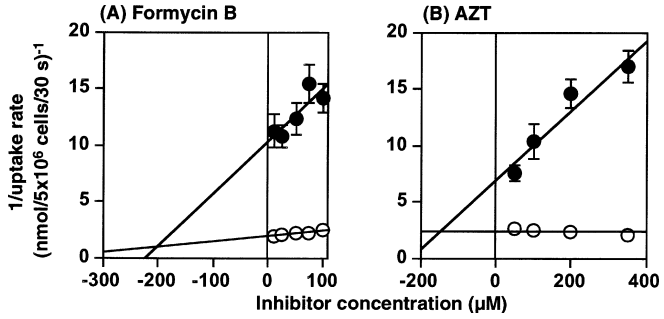

Fig. 5A, B *Cis*-inhibitory effects of formycin B and AZT on THP uptake by ENT-blocked Ehrlich cells in the presence of an inwardly directed Na^+ gradient. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 20 min in choline buffer, they were incubated with 1 μ*M* (●) or 5 μ*M* (○) THP and the indicated concentrations of formycin B or 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. Each point represents the mean ± SE from three experiments. The Ki values of formycin B and AZT for THP uptake by Ehrlich cells were calculated to be 202 and 138 μ*M*, respectively

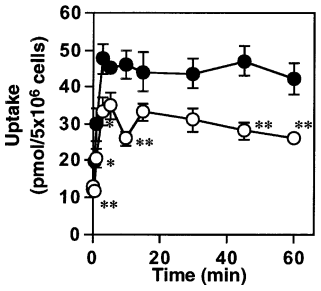

Fig. 7 Time courses of [^{3}H]uridine uptake by ENT-blocked Ehrlich cells in the presence or absence of an inwardly directed Na^+ gradient. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 20 min in choline buffer, they were incubated with 5 μ*M* uridine in sodium (●) or choline (○) buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for the indicated times at 37°C. Each point represents the mean ± SE from four experiments. $*P < 0.05$, $**P < 0.01$, vs the respective value in the presence of an inwardly directed Na^+ gradient at the corresponding time point

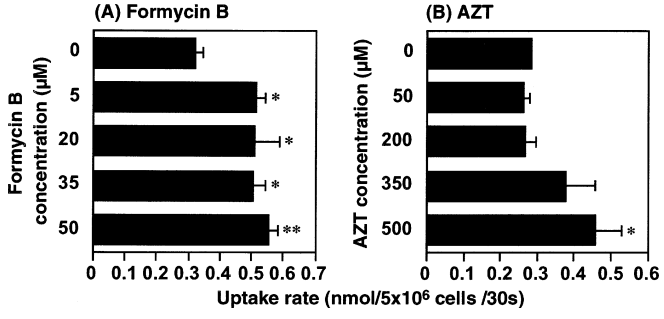

Fig. 6A, B *Trans*-stimulatory effects of formycin B and AZT on THP uptake by ENT-blocked Ehrlich cells in the presence of an inwardly directed Na^+ gradient. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and the indicated concentrations of formycin B or AZT for 15 min in choline buffer, 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. $*P < 0.05$, $**P < 0.01$, vs the respective control

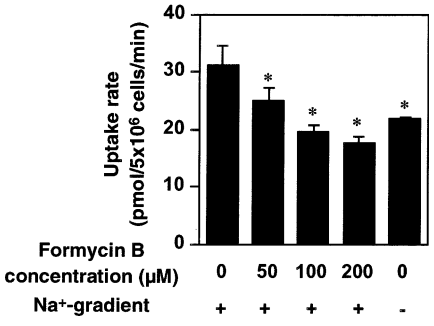

Fig. 8 Inhibitory effect of formycin B on [^{3}H]uridine uptake by ENT-blocked Ehrlich cells. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 20 min in choline buffer, they were incubated with 5 μ*M* uridine and the indicated concentrations of formycin B in sodium or choline buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 1 min at 37°C. Each point represents the mean ± SE from three experiments. $*P < 0.05$ vs the value without formycin B in the presence of an inwardly directed Na^+ gradient

by formycin B and AZT with apparent inhibition constants (Ki) of 201.9 and 138.0 μ*M*, respectively.

As can be seen in Fig. 6, the uptake of THP significantly increased with increasing preloaded amounts of formycin B or AZT. Thus, THP, formycin B and AZT are taken up by Ehrlich cells via a common system, although formycin B and AZT are recognized as substrates by other transporter(s).

Characteristics of [^{3}H]uridine uptake

To confirm the expression of CNT in the Ehrlich cells used in this study, the time course of [^{3}H]uridine uptake by ENT-blocked Ehrlich cells in the presence or absence of an inwardly directed Na^+ gradient was examined (Fig. 7). The uptake of [^{3}H]uridine in the absence of a Na^+ gradient was significantly less than that in the presence of a Na^+ gradient, but the uptake associated with the Na^+ gradient-dependent component was up to 30% of the total uptake.

The effect of formycin B, a representative ligand of CNT2, on the uptake of [^{3}H]uridine by Ehrlich cells is shown in Fig. 8. The rate of uptake of [^{3}H]uridine decreased with the addition of formycin B dose-dependently, the decreased uptake being approximately equal to the level in the absence of the gradient. So the Na^+ gradient-dependent uptake of [^{3}H]uridine is thought to be partially mediated by CNT2, although the expression of functional CNT2 in Ehrlich cells may be low.

The uptake of [^{3}H]uridine was significantly inhibited by the addition of THP (Fig. 9). The maximum inhibition was obtained at 100 μ*M* and did not change with increasing THP concentrations up to 200 μ*M*. The maximal THP-inhibited [^{3}H]uridine uptake in the

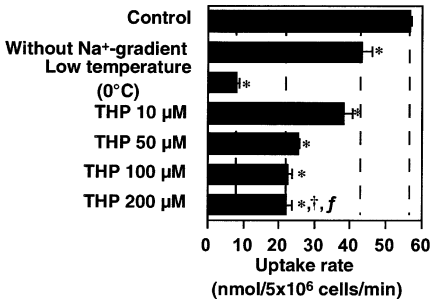

Fig. 9 Effect of THP on [^{3}H]uridine uptake by ENT-blocked Ehrlich cells. After cells had been pretreated with 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 20 min in choline buffer, they were incubated with 5 μ*M* uridine and the indicated concentrations of THP in sodium or choline buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 1 min at 0 or 37°C. Each point represents the mean ± SE from three experiments. $^*P < 0.001$ vs the control value, $^\dagger P < 0.001$ vs the value without the Na^+ gradient, $^f P < 0.001$ vs the value at low temperature

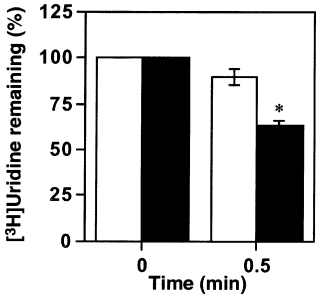

Fig. 10 *Trans*-stimulatory effect of THP on [^{3}H]uridine efflux from Ehrlich cells in the presence of an outward directed Na^+ gradient. After cells had been loaded with 5 μ*M* uridine for 15 min in sodium buffer containing 10 m*M* AZ plus 10 m*M* DOG, they were incubated with (*open bars*) or without (*closed bars*) 25 μ*M* THP in choline buffer containing 10 m*M* AZ plus 10 m*M* DOG and 10 μ*M* NBMPR for 30 s. Each bar represents the mean ± SE from five to six experiments. $^*P < 0.05$ vs the THP-untreated group

presence of an inwardly directed Na^+ gradient by ENT-blocked Ehrlich cells was less than that in the absence of the gradient. Furthermore, the uptake of [^{3}H]uridine by ENT-blocked Ehrlich cells at 0°C was much less than that inhibited by 200 μ*M* THP at 37°C. These results suggest that uridine uptake by Ehrlich cells is mediated by at least three systems other than ENT, that is, CNT2, a Na^+-independent THP-sensitive system, and a Na^+-independent THP-insensitive system.

Figure 10 shows the *trans*-stimulatory effect of THP on the efflux of [^{3}H]uridine from Ehrlich cells. The [^{3}H]uridine efflux from cells in the presence of an outwardly directed Na^+ gradient was significantly accelerated by the addition of 25 μ*M* THP to the extracellular space. Also, the [^{3}H]uridine uptake by THP-loaded Ehrlich cells in the presence of an inwardly directed Na^+ gradient increased by 15% (data not shown). Thus, THP and uridine indicated to be transported by the same transporter, probably CNT2, in Ehrlich cells.

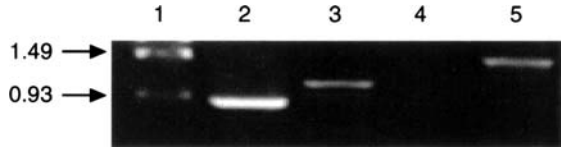

Fig. 11 Expression of mRNAs for CNT2 and CNT3 in Ehrlich cells determined by RT-PCR analysis. Total RNA was extracted as described in Material and methods and used for RT-PCR. Each lane was loaded with 25 μl of PCR product (*lane 1* size markers, *lane 2* CNT2 with RT, *lane 3* CNT2 without RT, *lane 4* CNT3 with RT, *lane 5* CNT3 without RT)

Expression of mRNAs for CNT isoforms in Ehrlich cells

The expression of CNT isoforms in Ehrlich cells was investigated by RT-PCR analysis. Expression of mRNA in Ehrlich cells was analyzed using primer sets specific to CNT2 and CNT3 as described in Materials and methods. As shown in Fig. 11, the message for CNT2 was detected, but not that for CNT3, and the sequence of the PCR product obtained showed 99.9% identify with CNT2. Furthermore, message was not detected in the case of the three different primer sets specific to CNT3 (data not shown). Thus, among the CNT isoforms, CNT2, but not CNT3, was expressed in the Ehrlich cells used in this study, and can be considered to be involved in the uptake of THP and uridine.

Discussion

There have been many reports on NT in various animal cells [11]. ENT can be classified into two subtypes, NBMPR-sensitive (*es*, NBMPR Ki < 1 μ*M*) and NBMPR-insensitive (*ei*, NBMPR Ki > 1 μ*M*) systems. At least five classes of CNT isoforms are known: CNT1/*cit*, which is concentrative, NBMPR-insensitive, and selective for pyrimidine and adenosine; CNT2/*cif*, which is concentrative, NBMPR-insensitive, and selective for purines, formycin B and uridine; CNT3/*cib*, which is concentrative, NBMPR-insensitive, and exhibits a broad selectivity toward purines, pyrimidines and their analogs, CNT4/*cit-like*, which recognizes guanosine as a permeant; and *cs*, which is concentrative and sensitive to inhibition by low nanomolar concentrations of NBMPR [5, 11, 20]. In this study, we clarified the involvement of NT and identified its isoform in anthracycline uptake by mouse Ehrlich cells.

The uptake of both THP and DOX in the absence, but not in the presence, of an inwardly directed Na^+ gradient was inhibited by representative substrates/inhibitors for ENT, but the inhibition was extremely low (Fig. 2). So we consider that THP and DOX were taken up by ENT, although the contribution might be negligible under physiological conditions because there was no inhibitory effect of the representative inhibitors in the presence of an inwardly directed Na^+ gradient. More detailed studies are needed to clarify this.

The experiments shown in Figs. 3, 4, 5 and 6 demonstrated that the uptake of THP, but not that of DOX, by ENT-blocked Ehrlich cells required an inwardly directed Na^+ gradient as a driving force, was inhibited by representative nucleosides and their analogs, and was subject to *cis*-inhibitory and *trans*-stimulatory effects of nucleoside analogs. Thus, THP is transported by the same system as that for nucleoside, i.e. CNT, and Na^+-dependent THP uptake is fully explained by CNT-mediated transport. As shown in Figs. 7, 8 and 9, [^{3}H]uridine was partially taken up by ENT-blocked Ehrlich cells via formycin B-sensitive CNT. In addition, [^{3}H]uridine efflux from the cells was accelerated by the addition of THP to the extracellular compartment (Fig. 10), and this finding clearly rules out the possibility that the transporter-like characteristics of THP uptake are due to the self-association of THP and/or the formation of a THP-uridine complex [1]. That is, [^{3}H]uridine efflux from the intracellular compartment can be considered to be stimulated by the accelerated conformational change of the transporter on the addition of THP to the extracellular compartment.

Yao et al. consider that *trans*-acceleration is a powerful methodology to screen nucleoside analogs as CNT permeates [29]. We believe that this finding constitutes definitive kinetic evidence of transporter-mediated uptake of THP by Ehrlich cells, and the involvement of the same system, namely CNT, in the uptake of THP and uridine by Ehrlich cells. As described above, CNT2 was selective for formycin B, and the mRNA for CNT2, but not that for CNT3, was detected in Ehrlich cells (Fig. 11). Therefore, among the CNT isoforms, CNT2 was indicated to be the transporter responsible for THP uptake by Ehrlich cells. However, to obtain definitive evidence, we are now establishing a CNT2-overexpressing mammalian cell line, and in the near future will perform a kinetic study using the transfectant. We cannot explain why DOX is not a substrate for CNT2 in Ehrlich cells, although it might be because of the difference between its chemical structure and that of THP, THP being a (2R″)-4′-*O*-tetrahydropyranyl-derivative of DOX (Fig. 1), and because of the low expression level of the relevant CNT isoform in Ehrlich cells, in which the uridine uptake depended on the inwardly directed Na^+ gradient by up to 30% (Fig. 7).

We have previously reported that the contribution of transporter-mediated uptake to the total cellular uptake of THP by Ehrlich cells is 78% [15], and so, based upon this information and the findings of this study, the maximum contribution of CNT2 is estimated to be 54%. Judging from the findings obtained in Fig. 9, in addition to ENT and CNT2, [^{3}H]uridine uptake seems to consist of at least two components, a Na^+-independent, THP-sensitive system and a Na^+-independent, THP-insensitive system, and the former might contribute to THP uptake by Ehrlich cells. As one of the candidates, the organic anion transporter (OAT) family, especially OAT3 and OAT4, by which AZT is recognized as a substrate, may contribute to THP uptake by Ehrlich cells. We found that the same transporter mediated the uptake of THP and AZT, as shown in Figs. 5 and 6, and this transporter was thought not to be CNT2 because AZT is not a substrate for CNT2. Takeda et al. have found that the Michaelis constants for AZT uptake via human OAT3 and OAT4 are 145.1 and 151.8 μM, respectively [27], and these values are comparable to the Ki value of AZT for THP uptake by Ehrlich cells (138 μM). Thus, it is suggested that OAT3 and OAT4 might be involved in THP uptake, but detailed studies to examine the involvement of other transport system(s) involved in the uptake of THP remain to be performed.

In a previous study, we have demonstrated that the cytotoxicity of THP, at least in part, depends on the uptake efficiency of the transport system in Ehrlich cells, and in this study one of the transporters partially involved was indicated to be CNT, probably CNT2. So the uptake efficiency of THP via CNT2, at least in part, contributes to its cytotoxicity. In human uterus, ovary, prostate and lung, the expression of CNT2 has been reported to be increased in tumor cells compared with the respective normal cells [19]. The development of anthracyclines utilizing the characteristics of CNT2 may therefore lead to the enhancement of chemotherapeutic efficacy for such tumors.

In conclusion, the findings of this study suggest that (1) THP and DOX may be substrates for the ENT expressed in Ehrlich cells, but their contribution is negligible, and (2) uptake of THP, but not of DOX, is mediated by a uridine-transportable CNT in Ehrlich cells.

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