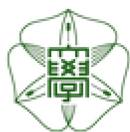

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Characterization of deoxyribonucleoside transport mediated by concentrative nucleoside transporters

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Abstract

Human concentrative nucleoside transporters (CNTs) are responsible for cellular uptake of ribonucleosides; however, although it is important to better characterize CNT-subtype specificity to understand the systemic disposition of deoxyribonucleosides (dNs) and their analogs, the involvement of CNTs in transporting dNs is not fully understood. In this study, using COS-7 cells that transiently expressed CNT1, CNT2, or CNT3, we investigated if CNTs could transport not only ribonucleosides but also dNs, i.e., 2′-deoxyadenosine (dAdo), 2′-deoxyguanosine (dGuo), and 2′-deoxycytidine (dCyd). The cellular uptake study demonstrated that dAdo and dGuo were taken up by CNT2 but not by CNT1. Although dCyd was taken up by CNT1, no significant uptake was detected in COS-7 cells expressing CNT2. Similarly, these dNs were transported by CNT3. The apparent K_m values of their uptake were as follows: CNT1, K_m = 141 μM for dCyd; CNT2, K_m = 62.4 μM and 54.9 μM for dAdo and dGuo, respectively; CNT3, K_m = 14.7 μM and 34.4 μM for dGuo and dCyd, respectively. These results demonstrate that CNTs contribute not only to ribonucleoside transport but also to the transport of dNs. Moreover, our data indicated that CNT1 and CNT2 selectively transported pyrimidine and purine dNs, respectively, and CNT3 was shown to transport both pyrimidine and purine dNs.

Keywords: deoxyribonucleoside; 2′-deoxyadenosine; 2′-deoxyguanosine; 2′-deoxycytidine; thymidine; concentrative nucleoside transporter

Introduction

Human concentrative nucleoside transporters (CNTs) have important physiological functions and are responsible for the cellular uptake of ribonucleosides, which act as signalling molecules [1]. The CNT family has three members (CNT1, CNT2, and CNT3) that contribute to nucleoside transport in a sodium-dependent manner. CNT1 and CNT2 are sodium-dependent carriers mainly for pyrimidine and purine nucleosides, respectively, whereas CNT3 has wide substrate selectivity and mediates the transport of nucleosides both in a sodium- and a proton-coupled manner [2–4]. CNTs are expressed broadly in various tissues such as enterocytes, hepatocytes, and kidneys, whereas expression of CNT3 has not been observed in the liver [5–8].

Because nucleic acids are synthesised in the body, their importance as nutrients has been overlooked. In recent years, the role of nucleic acids in physiological functions is being clarified, and the significance of ingesting dietary nucleotides and nucleosides is being reviewed. For example, salmon milt contains an abundance of deoxyribonucleotides (dNMPs) that are known as a component in dietary supplements containing nucleic acids. Nakamichi *et al* [9]. have reported that salmon milt extract might enhance brain function under normal conditions in mice, and nucleic acid may be a functional component contributing to this effect. In addition, Kojima-Yuasa *et al* [10]. have reported a protective effect of salmon milt extract against ethanol-induced liver injury in rats. In addition, although they reported that 2′-deoxyadenosine (dAdo) suppressed ethanol-induced cell death in primary culture of rat hepatocytes, they could not identify components that showed effect *in vivo*. The effects of orally ingested nucleic acids are assumed to be exerted after their absorption from the gastrointestinal tract followed by distribution to target tissues, but the disposition (including intestinal absorption and distribution) of dietary nucleic acids, particularly dNMPs, is not fully

understood. Our previous study demonstrated that CNT3 mediates the intestinal absorption of dAdo from 2′-deoxyadenosine 5′-monophosphate (dAMP) along with ecto-5′-nucleotidase [11]. However, the involvement of CNT3 in transporting other deoxyribonucleosides (dNs) such as 2′-deoxyguanosine (dGuo) and 2′-deoxycytidine (dCyd) remains unclear. In addition, it is not clear whether other members of CNT family, i.e. CNT1 and CNT2, contribute to the transport of these dNs. Therefore, a comprehensive characterization of CNT substrates is needed to understand the intestinal absorption mechanism of dNs and their subsequent utilisation in body tissues.

The purpose of the present study is to elucidate the involvement of CNTs in the cellular uptake of dNs. To this end, we investigated whether CNTs can transport dNs (dAdo, dGuo, and dCyd) in COS-7 cells transiently expressing CNT1, CNT2, or CNT3.

Materials and methods

Chemicals

[2,8-^{3}H]2′-Deoxyadenosine ([^{3}H]dAdo; 250 µCi; 9.25 MBq; 32.2 Ci/mmol), [8-^{14}C]2′-deoxyguanosine ([^{14}C]dGuo; 10 uCi; 370 kBq; 54.5 mCi/mmol), and [6-^{3}H]2′-deoxycytidine ([^{3}H]dCyd; 250 µCi; 9.25 MBq; 6.2 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [Methyl-^{3}H]thymidine ([^{3}H]thymidine; 250 uCi; 9.25 MBq; 20 Ci/mmol) was purchased from Perkin Elmer (Boston, MA). S-(4-Nitrobenzyl)-6- thioinosine, non-radiolabelled dAdo, and dGuo were purchased from Wako Pure Chemical Industries (Osaka, Japan). All the other chemicals were purchased from Sigma (St. Louis, MO, USA). Non-radioactive dNs and S-(4-nitrobenzyl)-6-thioinosine were dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the final study medium was 0.1% in the presence or absence of inhibitors. All other reagents were of the highest grade available and were used without further purification.

Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and incubated at 37 °C in 5% CO_2. To establish cells expressing human CNT1 or CNT2, pcDNA3.1-CNT1 and pcDNA3.1-CNT2 were purchased from GenScript Japan (Tokyo, Japan). Human CNT3 cloned into the pcDNA3.1 vector was constructed as previously reported [11]. The previously published transfection protocol was used [11]. The uptake of dNs by the cells was examined 48 hours post-transfection. An empty vector (pcDNA3.1) was included as a control in all experiments.

Western blotting

Whole proteins were extracted from the cultured cells using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with 1 mM phenylmethylsulphonyl fluoride and protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan). The samples (30 μg) were resolved using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Western blot was carried out with antibodies against CNT1, CNT2, CNT3, and β-actin. The antibodies' information was listed as follows: CNT1 (ab192438, diluted 1:1000; Abcam, Cambridge, MA, USA), CNT2 (A16086, diluted 1:1000; ABclonal, Wuhan, China), CNT3 (ab223085, diluted 1:1000; Abcam, USA), β-actin (ab179467, diluted 1:1000; Abcam, USA), and anti-rabbit secondary antibody (diluted 1:4000; Santa Cruz Biotechnology, Dallas, TX, USA).

Uptake experiment

The uptake of radiolabelled dNs by CNT1, CNT2, and CNT3 was evaluated using COS-7 cells transiently expressing each transporter as described previously [11]. Briefly, the cells were washed and pre-incubated with Hank's balanced salt saline (HBSS) buffer adjusted to pH 7.4 at 37 °C for 10 min. Subsequently, uptake was initiated by adding 0.5-mL HBSS containing radiolabelled substrate, and the cell monolayers were incubated for the indicated time at 37°C. Further, 10 μM S-(4-nitrobenzyl)-6-thioinosine (also known as NBMPR) was added in the HBSS buffer to inhibit the endogenous Na^+-independent nucleoside transporters. After the incubation, each cell monolayer was rapidly washed twice with 0.5-mL ice-cold HBSS. To quantify the radioactivity of radiolabelled substrate, the cells were solubilised in 1% SDS/0.2-N NaOH, and the resulting solution was mixed with 3 mL of scintillation cocktail (Perkin

Elmer, Waltham, MA) to estimate radioactivity. The radioactivity was quantified using a liquid scintillation counter. All the uptake values were corrected for protein content. Protein concentration was determined using a Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL), in accordance with the manufacturer's instructions.

Statistical and kinetic analysis

All data are expressed as mean ± standard error of the mean.

Nonlinear regression analysis was performed using SigmaPlot 12.5 (HULINKS). The kinetic parameters were calculated using the following equation:

$$V = V_{\mathrm{max}}\ [\mathrm{S}] / (K_{\mathrm{m}} + [\mathrm{S}])$$

where, V is the uptake rate of the substrate, V_{max} is the maximum uptake rate, [S] is the concentration of the substrate in the medium, and K_{m} is the Michaelis-Menten constant.

Results

Involvement of CNTs in cellular uptake of dNs

Western blotting analysis confirmed the protein expression of CNT1, CNT2, and CNT3 in COS-7 cells transiently expressing each transporter (Figure 1). To examine whether CNTs contribute to dN transport, the uptakes of $[^3H]$dAdo, $[^{14}C]$dGuo, $[^3H]$dCyd, and $[^3H]$thymidine by COS-7 cells transiently expressing CNT1 (COS-7/CNT1), CNT2 (COS-7/CNT2), or CNT3 (COS-7/CNT3) were measured. The $[^3H]$dAdo and $[^{14}C]$dGuo uptake by COS-7/CNT2 or COS-7/CNT3 was greater than that by COS-7/pcDNA3.1 and in a time-dependent manner, indicating that the compounds are substrates of CNT2 and CNT3 (Figures 2A and 2B). No significant uptake was observed in COS-7/CNT1. Uptake of $[^3H]$dCyd was greater in COS-7/CNT1 and COS/CNT3 than in control cells while no significant difference was observed in the

uptake of [^{3}H]dCyd by COS-7/CNT2 and COS-7/pcDNA3.1 (Figure 2C). The uptake of [^{3}H]thymidine, a substrate for CNT1 and CNT3, was significantly increased in COS-7/CNT1 and COS-7/CNT3 but not in COS-7/CNT2 as compared with control cells (Figure 2D) [2,4,12].

[Figure 1 near here]

[Figure 2 near here]

Kinetic analysis of dN uptake by COS-7 cells transiently expressing CNTs

To determine the kinetic basis for the differential uptake of CNT1, CNT2, and CNT3, concentration-dependent uptake of dNs was conducted. Figures 3A and 3B showed that CNT1-mediated uptake of [^{3}H]dCyd and [^{3}H]thymidine was concentration dependent and saturable at higher concentrations. The values obtained for kinetic parameters are summarised in Table 1. As shown in Figures 3C and 3D, the uptake of [^{3}H]dAdo and [^{14}C]dGuo by CNT2 was saturable at higher concentrations, with an apparent K_m of 62.4 μM and 54.9 μM, respectively. Moreover, CNT3-mediated uptake of [^{14}C]dGuo, [^{3}H]dCyd and [^{3}H]thymidine was also saturable at higher concentrations, and their K_m values were estimated to be 14.7, 34.4, and 52.7, respectively (Figures 3E-3G). In addition, previous experiments by our group demonstrated that the CNT3-mediated uptake of dAdo was also saturable, with an apparent K_m of 56 μM [11].

[Figure 3 near here]

[Table 1 near here]

Discussion

In general, dietary nucleic acids are believed to be digested to nucleotides in the intestinal lumen followed by dephosphorylation and absorption of nucleosides from the intestine [13]. Little is known about the mechanism of intestinal absorption of dNs found in DNA, and ribonucleosides have been shown to be substrates for multiple nucleoside transporters expressed in the small intestine. We previously reported that the intestinal absorption of dAdo from dAMP in Caco-2 cells was mediated at least partly by CNT3; Caco-2 cells are considered to be a cellular model of the human intestinal epithelium [11]. However, other CNT family members, namely CNT1 and CNT2 are expressed on the apical membrane of human enterocytes; it is thus difficult to evaluate comprehensively CNTs-mediated transport of dNs by using the Caco-2 cell model because of the absence of both transporters in these cells [14]. In this study, using a transiently transfected cell culture system, we investigated whether or not each CNT can transport not only ribonucleosides but also dNs. This is the first comprehensive characterization of dN transport in which dAdo, dGuo, and dCyd were found to be substrates for CNTs.

First, we examined whether each CNT substrate was involved in mediating dN uptake in COS-7 cells transiently expressing CNT1, CNT2, or CNT3 (Figure 2). The uptake of all dNs tested by COS-7/CNT3 cells was obviously higher than that by the control cells, indicating that CNT3 can contribute to the transport of both purine and pyrimidine dNs. In addition, purine dNs (dAdo and dGuo) were taken up by CNT2, whereas pyrimidine dNs (dCyd and thymidine) were taken up by CNT1. These results indicate that the substrate selectivity of each subtype for dNs is similar to that existing for ribonucleosides. As shown in Figure 2A, uptake of dAdo by CNT1 tended to be higher than that by control cells but was not considered to be significant. Ritzel *et al*. [2]

have reported that CNT1 can transport dAdo in Xenopus oocyte expression systems, although the CNT1-mediated transport of dAdo is very slow compared with that of adenosine. Additionally, CNT2 has been reported to transport cytidine despite of its purine-nucleoside selectivity; however, the CNT2-mediated transport of dCyd was not detected in our experiments [12]. Likewise, CNT3-mediated transport of dAdo was shown to be less than that of adenosine [15]. These results indicate that CNT-mediated transportability of dNs is lower than that of ribonucleosides; it further suggests that 2′-hydroxyl group of the ribose moiety plays a key role in the transport and recognition of nucleosides by CNTs. Moreover, the replacement of 2′-hydroxyl group by hydrogen might provide strict substrate selectivity for pyrimidine and purine dNs by CNT1 and CNT2, respectively.

To investigate the kinetic properties of CNTs-mediated transport of dNs, we examined the concentration-dependent uptake of dNs. CNT-mediated uptake of all dNs tested was saturable and was described by the Michaelis-Menten equation (Figure 3 and Table 1). Apparent K_m values of thymidine uptake by CNT1 and CNT3 were in the micromolar range and were similar to K_m values reported previously [16,17]. The K_m value of dAdo uptake by CNT2 was close to that by CNT3, and the transport efficiency (V_{max}/K_m) for dAdo by CNT2 was much lower than that by CNT3. This observation is consistent with previous studies demonstrating that dAdo is a poor permeant of CNT2 and that CNT3 concentrates substrates intracellularly more efficiently than other CNTs [5,18]. dGuo and dCyd analogues, such as entecavir and gemcitabine, are transported by several transporters, including CNTs; however, little has been reported on the characterization of transport of dN(s) [19,20]. Cropp *et al.* [21] reported that organic anion transporter 2 (OAT2) transported dGuo (K_m = 128 μM). Moreover, the affinity to CNT2 and CNT3 (K_m = 54.9 ± 14 μM and 14.7 ± 3.7 μM respectively) was higher

than that to OAT2. Given that OAT2 is mainly expressed in the liver, its contribution to intestinal absorption of dGuo might be negligible [22]. dCyd is transported by equilibrative nucleoside transporters that facilitate diffusion of nucleosides [23]. The present study demonstrated that dCyd is also transported by CNT1 and CNT3. The transport efficiency for dCyd by CNT3 was 10-fold higher than that by CNT1. Overall, the transport efficiency of each dN tended to be higher for CNT3 than that of CNT1 or CNT2. Although this might be caused by the difference in transient expression efficiency between the three entities, it might be explained, at least partially, by CNT3 having a greater ability to transport nucleosides than the aforementioned CNTs [5]. A further investigation comparing their transport efficiency using cell systems that express these transporters at similar levels so that the V_{max} values are directly comparable with each other is required.

Previous studies by other groups have shown that CNT3 is the major CNT expressed in the apical membrane of kidney proximal tubular epithelial cells; conversely, it is expressed at a very low level or is not expressed in the liver [8,24–26]. Additionally, our previous study has shown that CNT3 is expressed all along the small intestine [11]. Given the acidic microenvironment of the duodenum, jejunum, and lumen of the proximal renal tubule, CNT3, which is also a proton acceptor, is likely to play a critical role in the intestinal absorption of dietary nucleic acid and the renal reabsorption of dNs [27,28]. In the liver, CNT1 and CNT2, but not CNT3, are located at the sinusoidal and canalicular membranes, suggesting that both the transporters handle pyrimidine and purine dNs, respectively [7,8]. Recently, Nakamichi *et al.* [9] reported plasma concentrations in mice of several nucleosides, including dCyd, after oral administration of salmon milt extract that contains high levels of dNMPs. Plasma concentrations in these group were higher than in the vehicle group. Although their

findings are limited by the fact that nucleotides are degraded or recycled, nucleosides may do reach the systemic circulation after oral ingestion via transport through CNTs and equilibrative nucleoside transporters or other mechanisms. Further investigations of dNs transport and metabolism are needed to obtain a more comprehensive understanding of the disposition of dNs and to elucidate the nutritional significance of dietary DNA.

In conclusion, we identified dAdo, dGuo, and dCyd as substrates of CNTs and demonstrated that CNTs contribute not only to ribonucleoside transport but also to dNs transport. The substrate selectivity for dNs of each CNT studied was similar to that for ribonucleosides; specifically, CNT1 and CNT2 selectively transported pyrimidine and purine dNs, respectively, and CNT3 was shown to transport both pyrimidine and purine dNs. These findings will help to understand the absorption and disposition of each dN after ingesting dietary DNA. It can potentially contribute to the elucidation of the nutritional significance of dietary nucleic acids for improving or maintaining physiological functions.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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Table 1

Summary of kinetic parameters for the uptake of dN substrates mediated by CNT1, CNT2, and CNT3

Transporter	Substrate	Kinetic parameters (± standard error of mean)		V_{max}/K_m	Reference
		K_m (μM)	V_{max} (pmol/mg protein/min)		
CNT1	dCyd	141 ± 24	268.5 ± 24.0	1.91	—
	Thymidine	187 ± 32	3515 ± 348	18.8	—
CNT2	dAdo	62.4 ± 11	460.1 ± 41.3	7.37	—
	dGuo	54.9 ± 14	651.4 ± 72.9	11.9	—
CNT3	dAdo	56.3 ± 7.0	7782 ± 476	138	[11]
	dGuo	14.7 ± 3.7	608.1 ± 36.1	41.4	—
	dCyd	34.4 ± 6.7	715.4 ± 46.7	20.8	—
	Thymidine	52.7 ± 17	2954 ± 363	56.1	—

K_m: Michaelis-Menten constant; V_{max}: maximum uptake rate.

The kinetic parameters for the uptake of each dN were calculated using the the Michaelis–Menten equation using the data in Figure 3. Each value represents the mean ± standard error of mean of three independent experiments performed in triplicate.

Figure legends

Figure 1

Western blot analysis of CNT1, CNT2, and CNT3 expression in COS-7 cells after transient transfection.

Western blot analysis was performed using total proteins isolated from the COS-7 cell at 48 h after transfection. β-actin was used as a loading control.

Figure 2

Time course of dN uptake by CNT1-, CNT2-, and CNT3-expressing COS-7 cells.

COS-7 cells transfected with pcDNA 3.1 construct expressing human CNT1 (closed circle), CNT2 (closed triangle), or CNT3 (closed square) were incubated for the indicated times at 37 °C and pH 7.4 with each radiolabelled dN (2-nM [^{3}H]dAdo [A], 200-nM [^{14}C]dGuo [B], 10-nM [^{3}H]dCyd [C], and 10-nM [^{3}H]thymidine [D]). The cells transfected with pcDNA3.1 empty vector (open circle) were used as control cells. Each point represents the mean ± standard error of mean of three independent experiments performed in triplicate.

Figure 3

Concentration-dependence of CNT1-, CNT2- and CNT3-mediated uptake of dNs.

(A), (B): Concentration-dependence of CNT1-mediated uptake of dCyd and thymidine. (C), (D): Concentration-dependence of CNT2-mediated uptake of dAdo and dGuo. (E), (F) and (G): Concentration-dependence of CNT3-mediated uptake of dGuo, dCyd, and thymidine. Each uptake assay was initiated by adding either radiolabeled dN or a mixture of radiolabeled and non-radiolabeled dN to each well. Cells were incubated at

37 °C and pH 7.4 for 10 min (except for thymidine (1 min)) with varying concentrations of dN. The specific uptake by each CNT (closed triangle) was calculated by subtracting its uptake by control cells (open circle) from that by COS-7 cells transiently expressing each CNT (closed circle). Each point represents the mean ± standard error of the mean of three independent experiments performed in triplicate.

Figure 1

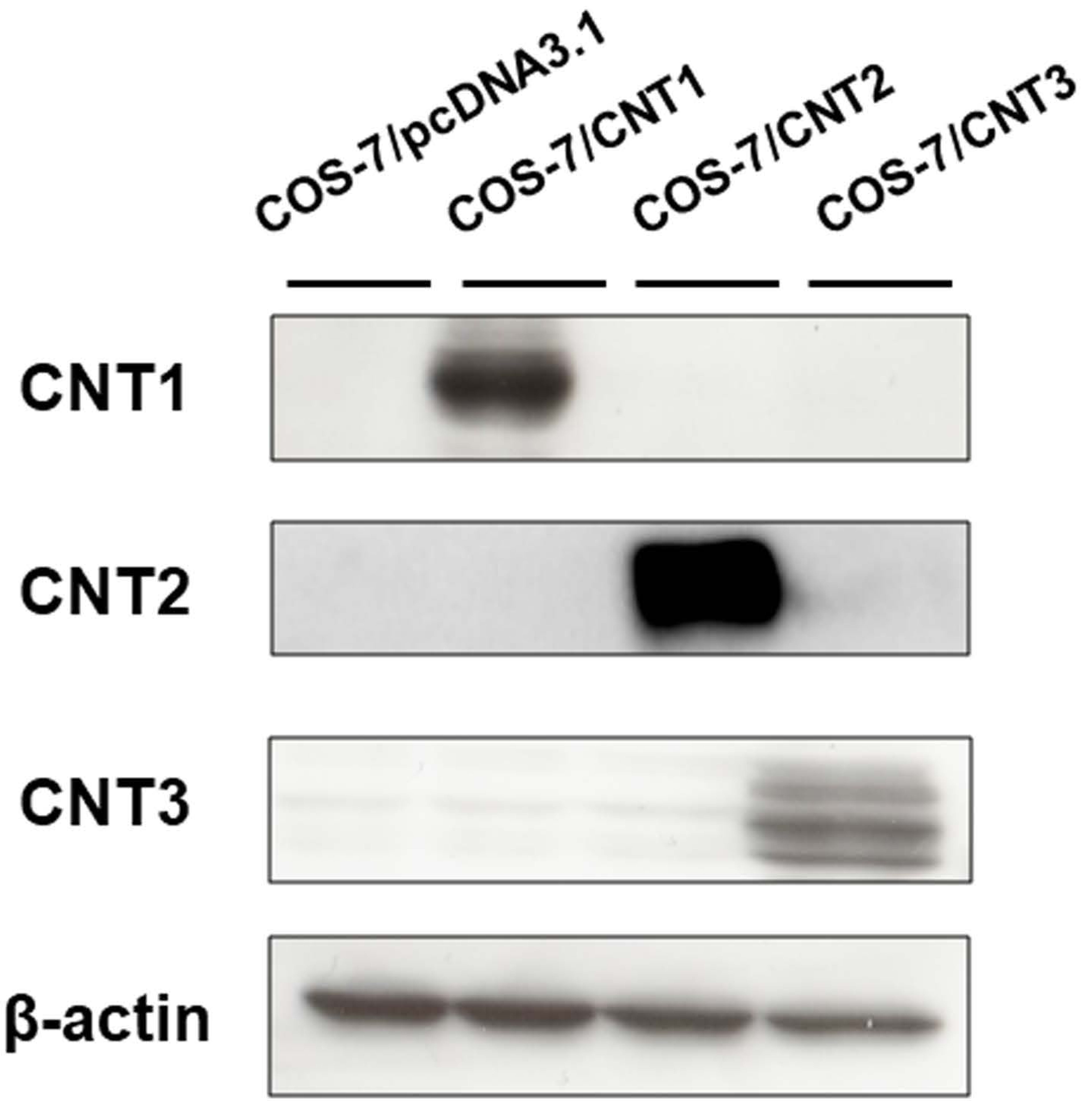
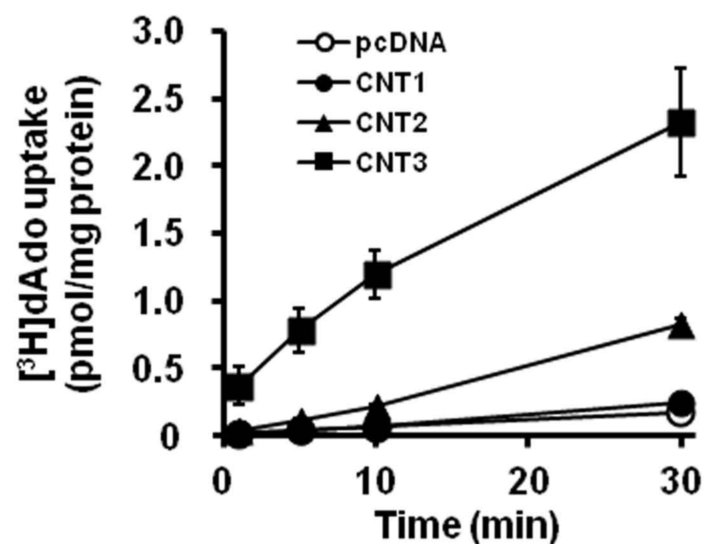
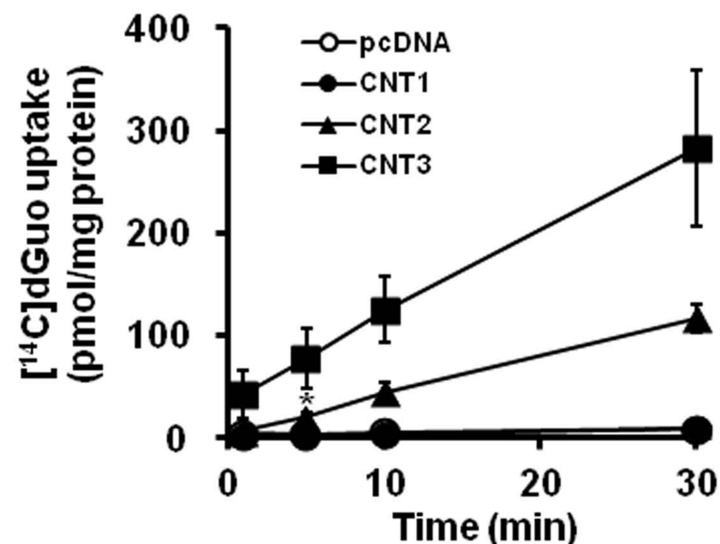
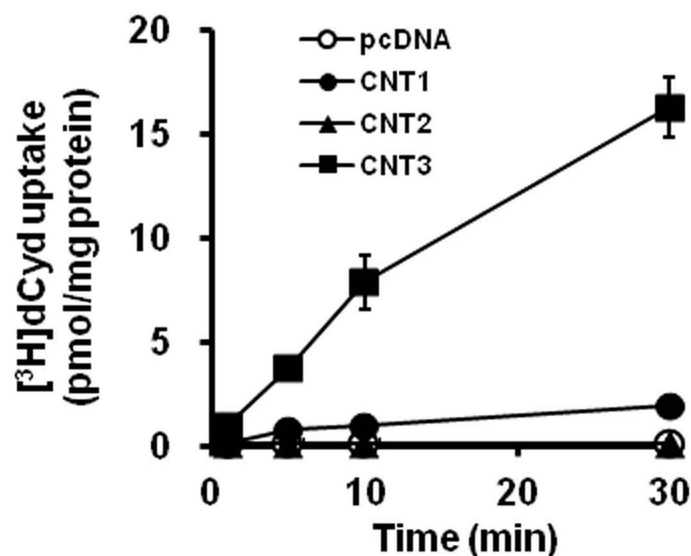


Figure 2







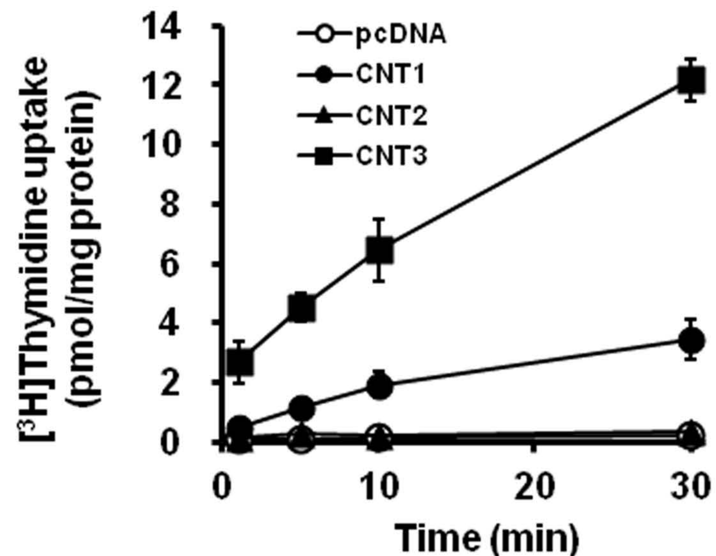


Figure 3