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Enzymatic synthesis and RNA interference of nucleosides incorporating stable isotopes into a base moiety

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ABSTRACT

Thymidine phosphorylase was used to catalyze the conversion of thymidine (or methyluridine) and uracil incorporating stable isotopes to deoxyuridine (or uridine) with the uracil base incorporating the stable isotope. These base-exchange reactions proceeded with high conversion rates (75–96%), and the isolated yields were also good (64–87%). The masses of all synthetic compounds incorporating stable isotopes were identical to the theoretical molecular weights via EIMS. ^{13}C NMR spectra showed spin–spin coupling between ^{13}C and ^{15}N in the synthetic compounds, and the signals were split, further proving incorporation of the isotopes into the compounds. The RNA interference effects of this siRNA with uridine incorporating stable isotopes were also investigated. A 25mer siRNA had a strong knockdown effect on the MARCKS protein. The insertion position and number of uridine moieties incorporating stable isotopes introduced into the siRNA had no influence on the silencing of the target protein. This incorporation of stable isotopes into RNA and DNA has the potential to function as a chemically benign tracer in cells.

1. Introduction

Recently, the behavior of intracellular RNA molecules has been the subject of a great deal of research.[1] mRNA plays a role in translating DNA information for the sequence of amino acids. The abundance and location of RNA in cells differs with the type of cell. Previous reports have shown that 20–25mer (short) sequences of double stranded RNA can inhibit the expression of a target protein,[2] and thus, the observation of RNA behavior is increasing in importance. Various highly sensitive fluorescent probe molecules/methods for tracing mRNA in cells have been developed, including: hybridization-sensitive molecules,[3] the molecular beacon method,[4] the probe molecule conjugated RNA binding protein,[5] the green fluorescent protein (GFP),[6] and the dye-binding light-up aptamer.[7] All are sensitive enough to allow detection of a single molecule, but are limited by their large size.

The intracellular environment is crowded-cells contain large numbers of big molecules, especially proteins, nucleic acids, and sugars.[8] The probes above (probes possessing a large labeled molecule) lack mobility and therefore cannot provide information concerning molecular mobility due to molecular crowding, and thus, cannot reflect the behavior of RNA in a cell accurately. Radioisotope labeling also has high sensitivity and is easily introduced into the termini of an RNA sequence at the 5′-^{32}P by T4 polynucleotide kinase. Radioisotope labeling does not change the size of the probe, however, a disadvantage is the risk associated with increased radiation exposure and the short half-life of some radioactive isotopes. A labeling method which does not change molecular size and does not cause damage is the stable isotope labeling method. Observation of molecules incorporating stable isotopes can be performed simply via mass spectrometry,[9] and imaging of biomaterial can be accomplished with isotope microscopy.[10]

In this research, stable isotopes were easily and rapidly introduced into the nucleoside chemical structure using a nucleic metabolic enzyme. Thymidine phosphorylase (TP) is a unique metabolic control enzyme that catalyzes the conversion of thymidine to deoxyribose-1α-phosphate and a free thymine base in a phosphate buffer.[11] Raap et al. tried to incorporate a stable isotope into the ribosyl position of a nucleoside with this enzyme, but the reaction yields were not high.[12] We previously developed a synthetic method involving TP to change the nucleobase moieties thymidine and various uracil derivatives substituted at the C5 position.[13] Herein is reported the enzymatic production of (deoxy)uridine, which facilitates the incorporation of stable isotopes including ^{13}C, ^{15}N, ^{18}O into a base moiety. Also reported is the use of labeled 5′-dimethoxytrithilated and 3′-phosphoramidated uridine in an automatic RNA synthesizer to introduce these tagged/labeled uridine moieties into an RNA sequence, and a comparison of the

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RNA interference effects of stable isotope-labeled and unlabeled siRNAs (Fig. 1).

2. Results and discussion

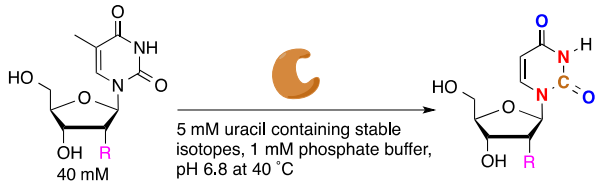
2.1. Base-exchange reaction with thymidine phosphorylase

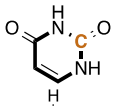
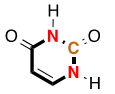
Initially, a base-exchange reaction was examined, using thymidine phosphorylase to synthesize deoxyuridine and uridine nucleosides incorporating stable isotopes. Starting substrates were either a ribosyl donor (such as thymidine or 5-methyluridine) or a uracil incorporating a ^{13}C, ^{15}N, or ^{18}O isotope. Reaction mixtures were composed of 40 mM thymidine (or 5-methyluridine), 5 mM of the labeled uracil compound, and 1 unit/mL of TP (from *Escherichia coli*; EC 2.4.2.4) in 1 mM phosphate buffer (pH 6.8), and were incubated at 40 °C. Product formation was monitored via UV absorption at 254 nm using HPLC with a C18 column (4.6 mm diameter, 5% MeCN in 1 mM phosphate buffer; pH 6.8). After removal of H_2O in vacuo, the residue was purified via preparative HPLC (reversed phase C18 column; 20 mm diameter, 5% MeCN in H_2O). Uridine possessing a uracil base with a stable isotope incorporated was eluted from the column. A combination of ^{1}H, ^{13}C, and ^{15}N NMR and mass spectrometric analysis was used to verify the structures of the various products as well as the incorporation of stable isotopes into the respective base moiety.

TP effectively converted (74.8–96.4%) the uracils incorporating various stable isotopes into the nucleoside (Table 1). The nucleoside isolated yields were slightly lower than the conversion ratios (63.5–87.4%), because purification was performed via repeated preparative HPLC operations. Neither the position of stable isotope incorporation, the identity of the converted stable isotope, or the number of stable isotopes incorporated had any relationship to TP reactivity. This result demonstrates the lack of steric hindrance on the uracil structure as a base. Of course, the atomic diameters of stable isotopes such as ^{12}C and ^{13}C, or ^{14}N and ^{15}N are effectively identical to the more common isotopes, and therefore, the chemical structure of a uracil moiety incorporating stable isotopes is the same as 'natural' uracil. In this study, the substrate specificity of TP did not change upon incorporation of stable isotope(s) into uracil.

The rate of base-exchange reaction for deoxyribose was faster than the reaction for the ribo form of uridine under the same conditions. In a previous report,[14] we noted that the absence of a 2′OH interfered with the enzymatic base-exchange reaction, and that the reaction time was extended. To reach equilibrium took about five times as long for the ribosyl form (20 to 24 h) as its deoxyribosyl form (4 h).

Table 1
Conversion and isolated yield of base exchange reactions using thymidine phosphorylase to convert thymidine to deoxyuridine possessing stable isotopes



Substrate	R = –H		R = –OH	
	Conv. (%)	Yield (%)	Conv. (%)	Yield (%)
	91.3	63.5	74.8	74.8
	96.4	87.4	78.9	64.2
	86.9	77.8	82.6	65.4
	84.9	69.8	77.9	75.6

C: ^{13}C, **N**: ^{15}N, **O**: ^{18}O.

2.2. Proof of existence of stable isotopes in synthetic compounds by mass spectrometry

Mass spectrometry is highly sensitive and capable of distinguishing between compounds incorporating only a few isotopes. The electron impact mass spectrometry (EI-MS) data presented in Table 2 makes it clear that stable isotopes have been introduced into the chemical structure of a deoxyuridine or ribo form of uridine. Increased molecular mass was observed in the synthetic nucleosides which resulted from the enzymatic base-exchange reaction. For instance, the molecular weight of 'normal' uridine is 244 by EI-MS, and the uridine incorporating one ^{13}C and two ^{15}N is 247 (+3, as predicted).

2.3. Proof of existence of stable isotopes in synthetic compounds by NMR

The ^{1}H NMR spectra of the natural compounds are identical to those of the synthetic deoxy-form and ribo-form compounds

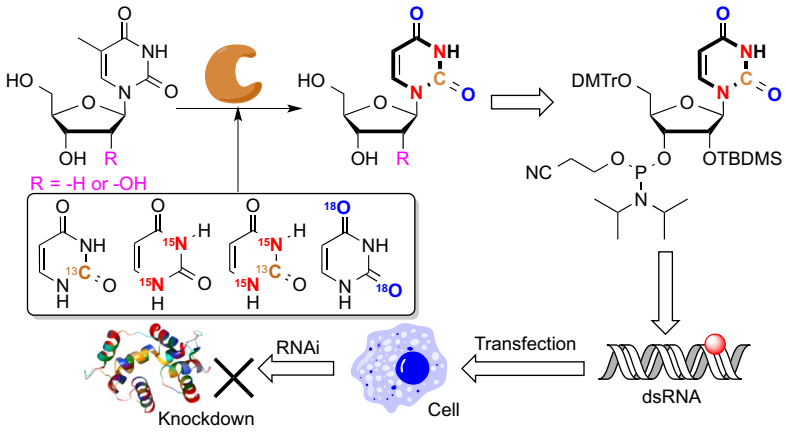
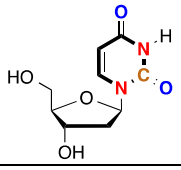
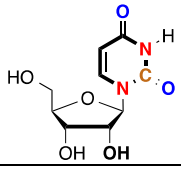


Figure 1. RNA interference by dsRNA containing the nucleosides incorporating stable isotopes and its production with thymidine phosphorylase.

Table 2
Comparison of predicted molecular masses of nucleosides incorporating stable isotopes with EI-MS data

Structure		**C** = ^{13}C	**N** = ^{15}N	**C** = ^{13}C **N** = ^{15}N	**O** = ^{18}O
Molecular weight	228.2	229.2	230.2	231.2	232.2
EI-MS	228	229	230	231	232
		**C** = ^{13}C	**N** = ^{15}N	**C** = ^{13}C **N** = ^{15}N	**O** = ^{18}O
Molecular weight	224.2	245.2	246.2	247.2	248.2
EI-MS	244	245	246	247	248

containing uridine(s) incorporating stable isotopes. On the other hand, ^{13}C NMR proved to be a very important tool for structural determination of compounds incorporating stable isotopes, as spectra drastically change depending on the isotope inserted. The results are shown in Figure 2. For example, 2-^{13}C deoxyuridine exchanges ^{12}C for ^{13}C at the 2 position of the base, resulting in the sharp singlet at 152 ppm (Fig. 2a). Because the natural isotopic abundance of ^{13}C is low (1.1%), a uracil with 99% ^{13}C substitution at the 2 position gives a remarkably large signal. (Natural abundance ^{13}C gives small signals for the 4 and 6 positions at 167 and 141, respectively.) Similarly, synthetic deoxyuridine introducing uracil possessing two different stable isotopes (2-^{13}C and 1,3-$^{15}N_2$) also shows a strong signal at 151 ppm. This compound has two nitrogen atoms with atomic weight 15 at the 1 and 3 positions of the deoxyuridine base, in addition to the ^{13}C substitution. There are two strong signals in the ^{15}N NMR (see Supplementary data), indicating successful incorporation of stable nitrogen isotopes into the deoxyuridine through the TP base-exchange reaction. In addition, the deoxyuridine 2-^{13}C is split into an overlapping doublet of doublets by the effectively magnetically non-equivalent neighboring ^{15}N (Fig. 2b). The signals for the carbons at the 4 and 6 positions are also split into doublets (Fig. 2b and c) by coupling with one ^{15}N neighbor each. Because of this, all signals were checked in detail, and the signals for C1′ and C5 in deoxyuridine incorporating ^{15}N were also split into doublets (long-range coupling; see Supplementary data). The signals derived from deoxyuridine introducing ^{18}O at the 2 and 4 positions (Fig. 2d) were nearly identical to those from 'normal' deoxyuridine. ^{13}C data from ribo-form uridine was fundamentally the same as for deoxyuridine (spectra available in Supplementary data).

2.4. RNA sequences and MALDI–TOF MS

RNA oligomers were synthesized using a commercially available synthesizer with phosphoramidite chemistry. Table 3 shows the synthetic ssRNA sequences used for RNAi experiments and the results of MALDI–TOF (matrix-assisted laser desorption/ionization time of flight) mass spectrometry analysis of the samples. We used the previously reported method to prepare the stable isotope-labeled phosphoramidite building blocks.[15] The 2′-*tert*-buthyldimethylsilyl, 3′-(2-cyanoethyl diisopropylphosphoramidite), and 5′-DMTr groups were introduced into 2-^{13}C, 1,3-$^{15}N_2$-uridine for automatic RNA syntheses (see Supplementary data). The ssRNAa and ssRNAc samples here utilize the same sequence; however, the uridines with stable isotopes occupy different positions in the sequence. The ssRNAa sequence has two uridines possessing stable isotopes near the middle (bold U* in Table 3), and sequence ssRNAc has one uridine incorporating stable isotopes at the center position. The 'a' and 'c' sequences have the

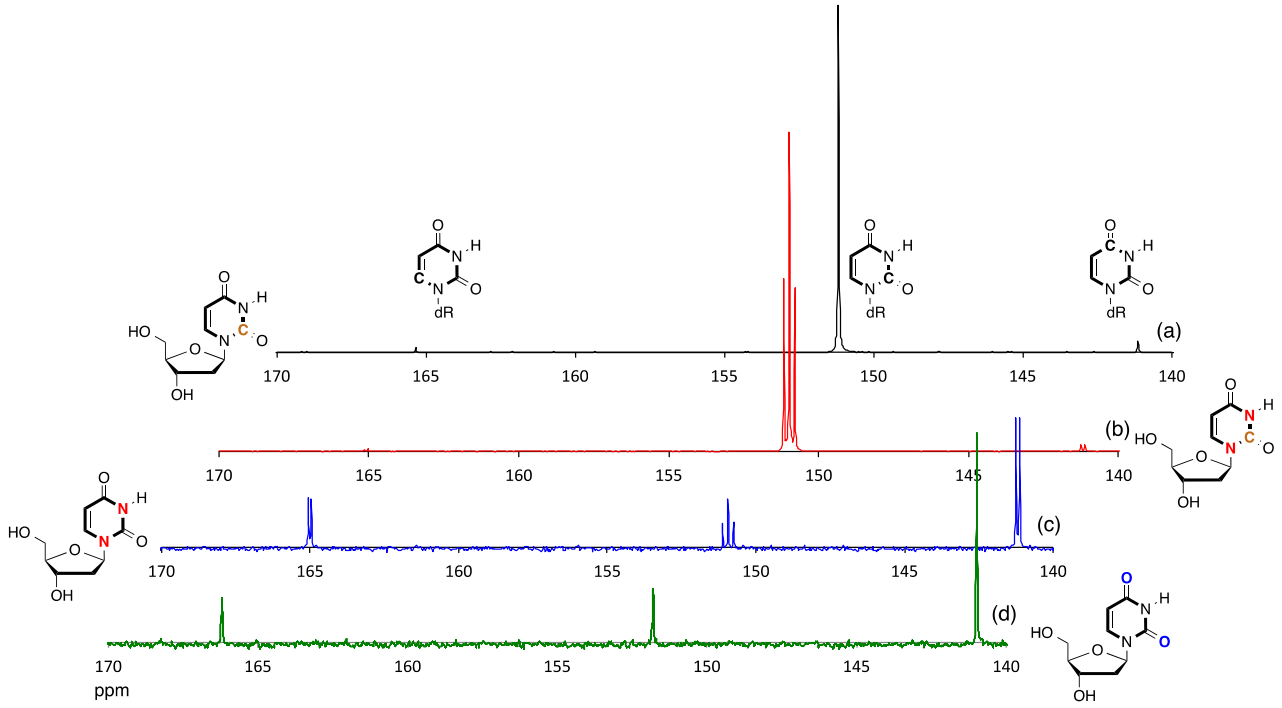


Figure 2. Comparison of ^{13}C NMR spectra for deoxyuridine incorporating stable isotopes. (a) 2-^{13}C-deoxyuridine, (b): 2-^{13}C, 1,3-$^{15}N_2$-deoxyuridine, (c): 1,3-$^{15}N_2$-deoxyuridine, (d): 2,4-$^{18}O_2$-deoxyuridine. The scale of the *y*-axis is different in each spectrum for clarity.

Table 3
Sequences and MS analysis of ssRNAs synthesized

Duplex RNA name	Single stranded RNA name	Sequence	Molecular weight (*m*/*z*)	
			Calculated	Observed
siRNA (SI1)	ssRNAa	5′-UUCGCUGCGG**U***C**U***UGGAGAACUGGG	8069	8068
	ssRNAb	3′-AAGCGACGCCAGAACCUCUUGACCC	7939	7939
siRNA (SI2)	ssRNAc	5′-UUCGCUGCGGUC**U***UGGAGAACUGGG	8058	8060
	ssRNAd	3′-AAGCGACGCCAGAACC**U***CUUGACCC	7950	7951
siRNA (SI0)	ssRNAe	5′-UUCGCUGCGGUCUUGGAGAACUGGG	—	—
	ssRNAb	3′-AAGCGACGCCAGAACCUCUUGACCC	—	—

U*: 2-^{13}C, 1,3-$^{15}N_2$ uridine.

same base order and as do ssRNAb and ssRNAd, which are composed of ribonucleosides having 0 or 1 labeled uridine in the sequences, respectively. The predicted molecular mass of each single-stranded 25 mer RNA matched well with that observed via MALDI–TOF MS (Table 3).

2.5. RNA interference

Next, the biological activity of labeled and unlabeled siRNAs was compared. To do so, the silencing effect of siRNAs targeting the myristoylated alanine-rich C-kinase substrate (MARCKS)[16] gene in the human neuroblastoma cell line SH-SY5Y was analyzed by western blotting. MARCKS plays an essential role in neuronal cell differentiation and central nervous system development.[17] It was found that 25mer, double-stranded RNAs suppress the expression of the target genes by causing cleavage of the mRNA, or inhibiting its translation (Fig. 3). β-Actin was used to revise errors between the columns in the western blot. The control experiment did not add the siRNA to the cells, and this did not suppress the knockdown of expression of the MARCKS protein (100%). When a transfection reagent such as RNAiMAX (Lipofectamine) was added without the siRNA, the silencing effect was moderate (28%, expression: 72%, see Supplementary data). On the other hand, a negative control siRNA proved to be less effective at interfering with expression of MARCKS (90%, Fig. 3, No. 2) due to a lack of interaction between the mRNA and dsRNA. Knockdowns were observed in RNA interference experiments using three 25mer, double-stranded siRNAs composed of the ssRNAs differing in the position of the labeled uridine discussed above (Table 3). Single-stranded sequences a and b (Table 3) were combined to form a double-stranded RNA (siRNA (SI1)), as were sequences c and d to form a complementary ds sequence with the labeled uracil in different positions (siRNA (SI2)). MARCKS siRNA (SI0), composed of one strand with the same sequence as ssRNAa (or c) and one strand with the same sequence as ssRNAb (or d), both having natural-abundance uracil, served as a positive control. SI0 had a strong silencing effect on the MARCKS protein (83%, expression: 27%, Fig. 3, No. 3). MARCKS siRNA (SI1), composed of ssRNAa and ssRNAb (incorporating two and zero labeled uridines, respectively, into the same sequences as for siRNA (SI0)), suppressed expression of the target protein even more (19%, Fig. 3, No. 4). MARCKS siRNA (SI2), composed of ssRNAc and ssRNAd (one labeled uridine in each ssRNA sequence), also showed the same effect (18%, Fig. 3, No. 5). Thus, the position and number of labeled uridine moieties does not appear to have an effect on the knockdown capability of the sequence.

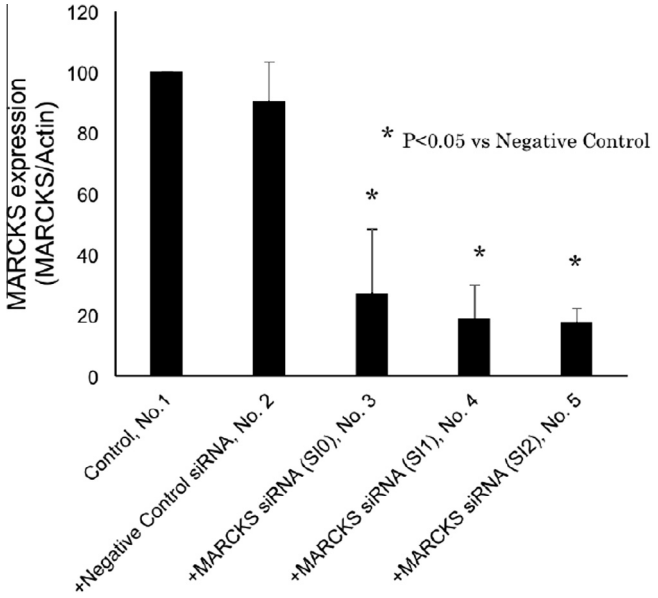


Figure 3. MARCKS knockdown effect of dsRNA with uridine incorporating stable isotopes (RNA interference). SH-SY5Y cells were treated with distilled water (control, No. 1), and non-targeting dsRNA (negative control siRNA, No. 2), normal dsRNA (MARCKS siRNA (SI0), 5′-UUCGCUGCGGUCUUGGAGAACUGGG: 3′-AAGCGACGCCAGAACCUCUUGACCC, No. 3), duplex RNA composed of ssRNAa and b inserting the labeled uridine into one chain of the dsRNA only (SI1, 5′-UUCGCUGCGG**U***C**U***UGGAGAACUGGG: 3′-AAGCGACGCCAGAACCUCUUGACCC, No. 4), and duplex RNA composed of ssRNAc and d inserting a labeled uridine into each chain of the dsRNA (SI2, 5′-UUCGCUGCGGUC**U***UGGAGAACUGGG: 3′-AAGCGACGCCAGAACC**U***CUUGACCC, No. 5). Experiments performed 4 times (see Supplementary data). Data show the expression of the MARCKS protein and concentration of standard β-actin of equal loading by western blotting. Data expressed as a percentage of total cells. Results shown as mean ± SEM (n = 3). *P <0.01 (Tukey's test).

3. Conclusion

Thymidine phosphorylase catalyzes the reaction of thymidine (or methyluridine) and uracil incorporating stable isotopes to form deoxyuridine (or uridine) with the uracil base incorporating the stable isotope. These base-exchange reactions occur with high conversion (75–96%), and the isolated yields are good (64–87%). EI-MS data for the synthetic compounds matched the theoretical molecular weights. While the ^{1}H NMR spectra of the synthetic compounds were identical to the natural-abundance compounds, the ^{13}C NMR spectra were different for each of the four compounds incorporating ^{13}C, ^{15}N and ^{18}O studied. Coupling between ^{13}C and ^{15}N in deoxyuridine or uridine was observed. 25mer siRNA sequences containing uridine incorporating stable isotopes showed as good or better knockdown effects on the MARCKS protein from SH-SY5Y cells as common siRNA for the MARCKS gene. The insertion position into and number of uridine incorporating stable isotopes in the siRNA had no influence on the silencing of the target protein. The insertion of stable isotopes into RNA and DNA has the potential to assist in examining the existence, mechanism of action, and location of signal expression in a cell.

4. Experimental section

4.1. General

All solvents and reagents were of reagent-grade quality, and used without further purification. Stable isotope reagents were of high purity (>95% isotope) and were supplied by Taiyo Nippon Sanso Corp. TLC analysis was carried out on silica gel 60 F_{254} 1.05554 (Merck). The ^{1}H, ^{13}C and COSY NMR spectra were recorded on a JEOL ECS 400 spectrometer (400.0 MHz for ^{1}H; 100.4 MHz for ^{13}C; 40.5 MHz for ^{15}N). Spectra were referenced to TMS in CD_3OD-d_4. Chemical shifts (δ) are reported in ppm. EI-MS was recorded on a GCMS-QP2010 Plus (Shimadzu). MALDI–TOF MS was recorded on a Voyager DE-STR (Applied Biosystems).

4.2. Enzymatic reactions

Thymidine phosphorylase (from *Escherichia coli*, EC 2.4.2.4) was purchased from Sigma–Aldrich Chemical Co. The units of enzymatic activity indicate the transition of native substrates as 1.0 μmol each of thymidine and phosphate to thymine and 2-deoxyribose-1-phophate per 1 min. In this research, a 'unit' refers to the amount of enzyme activity, even though both synthetic and natural substrates were in use.

Incubations generally contained 40 mM thymidine (0.4 mmol, 0.097 g), 5 mM various uracil derivatives (incorporated stable isotopes, 0.05 mmol) and 1 unit/mL of thymidine phosphorylase in 10 mL of 1 mM phosphate buffer (pH 6.8). Mixtures were stirred at 40 °C until the reactions reached equilibrium. Product formation was monitored via UV absorption at 254 nm using HPLC with a C18 column (4.6 mm diameter, 5% MeCN in 1 mM phosphate buffer; pH 6.8). After removal of H_2O in vacuo, the residue was purified using preparative HPLC (C-18 reverse phase column, 4.6 mm diameter, Unison UK-C18, Imtakt Co., Kyoto) by eluting with an H_2O-MeCN system (as above) to afford a pure nucleoside incorporating stable isotopes.

4.2.1. 2-^{13}C-uridine

^{1}H NMR (CD_3OD) δ 8.00 (1H, J = 8.0 Hz, d), 5.90 (1H, m), 5.69 (1H, J = 8.4 Hz, d), 4.18 (1H, m), 3.92 (1H, m), 3.73 (2H, m), 2.23 (1H, m). ^{13}C NMR (CD_3OD) δ 166.2, 152.1, 142.7, 102.6, 90.7, 86.3, 75.7, 71.3, 62.3. EIMS m/e 305 $[M]^+$.

4.2.2. 1,3-$^{15}N_2$-uridine

^{1}H NMR (CD_3OD) δ 7.97 (1H, J = 8.4 Hz, d), 6.26 (1H, J = 6.6 Hz, t), 5.69 (1H, m), 4.38 (1H, m), 3.92 (1H, m), 3.73 (2H, m), 2.23 (1H, m). ^{13}C NMR (CD_3OD) δ 166.3 (J = 9.5 Hz, d), 152.5 (J = 18, 18 Hz, dd), 142.7 (J = 12 Hz, d), 102.6 (J = 6.7 Hz, d), 90.6 (J = 11 Hz, d), 86.3, 82.5, 75.7, 71.2, 62.2. EIMS m/e 247 $[M]^+$.

4.2.3. 1,3-$^{15}N_2$-2-^{13}C-uridine

^{1}H NMR (CD_3OD) δ 7.97 (1H, m), 6.27 (1H, m), 5.71 (1H, m), 4.39 (1H, m), 3.94 (1H, m), 3.75 (2H, m), 2.25 (1H, m). ^{13}C NMR (CD_3OD) δ 166.3 (J = 11 Hz, d), 152.3 (J = 18, 18 Hz, dd), 142.5 (J = 12 Hz, d), 102.6 (J = 5.8 Hz, d), 88.9, 86.6 (J = 12 Hz, d), 72.2, 62.8, 41.3. ^{15}N NMR (CD_3OD) δ 152.2 (J = 18 Hz, d), 143.8 (J = 19 Hz, d). EIMS m/e 246 $[M]^+$.

4.2.4. 2,4-$^{18}O_2$-uridine

^{1}H NMR (CD_3OD) δ 8.00 (1H, J = 8.0 Hz, d), 5.90 (1H, J = 4.4 Hz, d), 5.71 (1H, m), 4.16 (1H, m), 4.01 (1H, m), 3.79 (2H, J = 2.8, 12, 46 Hz, ddd). ^{13}C NMR (CD_3OD) δ 166.2, 152.4, 142.7, 102.7, 90.6, 86.3, 75.7, 71.3, 62.2. EIMS m/e 248 $[M]^+$.

4.2.5. 2-^{13}C-deoxyuridine

H NMR (CD_3OD) δ 7.94 (1H, J = 3.2, 3.2, 8.0 Hz, ddd), 6.24 (1H, J = 6.2 Hz, t), 5.67 (1H, J = 3.4, 8.0 Hz, dd), 4.35 (1H, m), 3.89 (1H, m), 3.72 (2H, J = 3.5, 12, 25, 25 Hz, dddd), 2.21 (2H, m). ^{13}C NMR (CD_3OD) δ 165.0 (J = 9.5 Hz, d), 151.0 (J = 7.3 Hz, t), 141.1 (J = 12.4 Hz, d), 101.3 (J = 5.7 Hz, d), 87.6, 85.2 (J = 12.4 Hz, d), 70.9, 61.5, 40.0. ^{15}N NMR (CD_3OD) δ 148.6 (J = 16.8, 339 Hz, dd). EIMS m/e 229 $[M]^+$.

4.2.6. 1,3-$^{15}N_2$-deoxyuridine

^{1}H NMR (CD_3OD) δ 7.98 (1H, J = 1.6, 8.4 Hz, dd), 6.27 (1H, J = 6.6, 6.6 Hz, dd), 5.69 (1H, m), 4.30 (1H, m), 4.38 (1H, J = 3.2, 3.2, 3.2, 3.2 Hz, dddd), 3.69 (2H, m), 3.92 (1H, J = 3.2, 6.8 Hz, dd), 3.74 (3H, J = 3.4, 12, 29 Hz, ddd), 2.24 (2H, m). ^{13}C NMR (CD_3OD) δ 165.3, 151.1, 141.1, 101.3, 87.6, 85.3, 70.9, 61.5, 40.0. EIMS m/e 305 $[M]^+$.

4.2.7. 1,3-$^{15}N_2$, 2-^{13}C-deoxyuridine

H NMR (CD_3OD) δ 7.95 (1H, J = 1.9, 1.9, 8.0 Hz, ddd), 6.24 (1H, J = 6.8, 6.8 Hz, dd), 5.67 (1H, J = 2.8, 4.8, 7.6 Hz, ddd), 4.35 (1H, J = 3.2, 3.2, 3.4, 3.4 Hz, dddd), 3.89 (1H, J = 3.2, 6.8 Hz, dd), 3.72 (2H, J = 3.2, 12, 25 Hz, ddd), 2.21 (2H, m). ^{13}C NMR (CD_3OD) δ 165.0 (J = 9.5 Hz, d), 151.0 (J = 7.3, 7.3 Hz, dd), 141.1 (J = 12.4 Hz, d), 101.3 (J = 5.7 Hz, d), 87.6, 85.2 (J = 12.4 Hz, d), 70.9, 61.5, 40.0. ^{15}N NMR (CD_3OD) δ 148.6 (J = 16.8, 339 Hz, dd), 140.2 (J = 18.6, Hz, d). m/e 231 $[M]^+$.

4.2.8. 2,4-$^{18}O_2$-deoxyuridine

^{1}H NMR (CD_3OD) δ 7.98 (1H, J = 8.0 Hz, d), 6.30 (1H, J = 6.8 Hz, t), 5.72 (1H, J = 8.4 Hz, d), 4.40 (1H, m), 3.95 (1H, m), 3.79 (2H, m), 3.37 (1H, s), 2.29 (2H, m). ^{13}C NMR (CD_3OD) δ 166.2, 151.8, 141.0, 101.4, 87.6, 85.3, 70.9, 61.5, 40.0. EIMS m/e 232 $[M]^+$.

4.3. RNA interference

Commercially synthesized siRNA for human MARCKS and non-targeting siRNA were purchased from the Invitrogen and Bex corporations. Transfection of siRNA (10 nM) was performed using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. After 72 h of transfection, western blotting was carried out.

SH-SY5Y cells, a human neuroblastoma cell line, were grown in DMEM containing 10% fetal bovine serum (Sigma) at 37 °C in a humidified 5% CO_2 atmosphere. One day before the experiments, the cells were seeded at a density of 7×10^4 cells/cm^2 for western blotting.

4.4. Western blotting[17,18]

Western blotting was performed according to established procedures. After blocking with 0.5% skim milk in TBS-T (0.14 M NaCl, 0.01 M Tris pH 7.4, 0.1% Tween 20), the PVDF membranes were incubated with primary antibody solution containing anti-MARCKS antibody (1:3000 in TBS-T) for 60 min at room temperature. HRP-conjugated anti-goat IgG antibody (1:5000) was used as a secondary antibody. Cell homogenates were subjected to western blot analysis for MARCKS expression using Image J software. β-Actin was detected to monitor equal loading.

4.5. Statistical analysis[17]

Results are expressed as the mean ± SEM (standard error of the mean). Statistical differences between means were evaluated by Student's t-test or one-way ANOVA followed by Tukey's test and considered significant at P <0.05.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.09.011.

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