Synthesis of *N*-Acetylglucosamine-Modified ara-C and Its Effect on Ovarian Cancer Cells

Hiroshi Fujimoto, Tsutomu Takayanagi,† and Katsumi Ajisaka*

Meiji Institute of Health Science, 540 Naruda, Odawara 250, Japan

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1-β-D-Arabinofuranosylcytosine (ara-C) was modified by reaction of tetra-*N*-acetylchitotetraose ($(GlcNAc)_4$) using the transglycosylation activity of thermostable chitinase (EC 3.2.1.14) from *Bacillus licheniformis* X-7u. The structure of the modified ara-C was determined to be either β1-3′- or β1-5′-linked GlcNAc-ara-C or $(GlcNAc)_2$-ara-C. The total yield of these glycosylated ara-Cs was about 10%. GlcNAc-ara-C and $(GlcNAc)_2$-ara-C depressed the growth of G-401 cancer cells, while 5-*O*-β-D-galactopyranosyl-β-D-arabinofuranosylcytosine (Gal-ara-C) had no effect on G-401 cells.

Introduction

In certain cancer cells, the activity of extracellular glycosidases β-*N*-acetylglucosaminidase (EC 3.2.1.30), β-*N*-acetylgalactosaminidase (EC 3.2.1.53), and α-L-fucosidase (EC 3.2.1.51) has been observed to increase to a level severalfold as great as that of normal cells.[1-3] In particular, ovarian cancer cells displayed a remarkable increase in the amount of extracellular β-*N*-acetylglucosaminidase.

Although 1-β-D-arabinofuranosylcytosine (ara-C) has been shown to be an effective anticancer drug, it also exhibits strong side effects.[4,5] Consequently, many ara-C derivatives of lower toxicity, anhydro-, behenoyl-, or palmitoyl-ara-C, have been synthesized.[6-8] We started to survey for a new anticancer drug on the basis of the following concept. If ara-C could be modified by addition of GlcNAc, the linkage between GlcNAc and ara-C should be readily hydrolyzed by β-*N*-acetylglucosaminidase excreted by cancer cells. In such a case, the toxicity of ara-C is expected to be selective toward cancer cells. In other words, while the GlcNAc-modified ara-C might not express a toxicity toward either normal or cancer cells, the ara-C liberated by hydrolysis with β-*N*-acetylglucosaminidase around cancer cells would display the usual anticancer activity.

We have reported that thermostable chitinase from *Bacillus licheniformis* X-7u transferred the di-*N*-acetylchitobiose, $(GlcNAc)_2$, unit from the nonreducing end of tetra-*N*-acetylchitotetraose, $(GlcNAc)_4$, to hexa-*N*-acetylchitohexaose, $(GlcNAc)_6$.[9] We used the enzyme for the glycosylation of ara-C by the use of its transglycosylation activity.

The object of the present study was to synthesize the GlcNAc-modified ara-C and to examine its selective anticancer effects.

Results and Discussion[10]

Glycosylation of ara-C. Glycosylation of nucleoside derivatives with β-galactosidase or cellulase (EC 3.2.1.4) has been reported by Karasev et al.[11,12] 5-*O*- and 3-*O*-β-D-galactopyranosyl-β-D-thymidine and β-D-glucopyranosyl-β-D-thymidine were formed in the enzymatic transglycosylation reaction. In the present study, ara-C was used as a model compound for possible nucleoside anticancer drugs. A mixture consisting of 5% $(GlcNAc)_4$, 20% ara-C, and chitinase (0.66 unit) was incubated at 35 °C for 6 h. After heat denaturation of chitinase, the solution was loaded onto a Sephadex G-25 column (Figure 1). The peaks I and II were eluted at a faster rate than ara-C. The peaks I and II were further separated by HPLC attached with an Asahipak NH2P-50 column (Figure 2). From peak I, new peaks of A and B were obtained, and from peak II, new peaks of C and D were obtained. From the ^{13}C NMR analyses of peaks A–D, the structures of the four corresponding compounds were determined. In the spectrum of peak A (Table 1), the C-3′ signal of ara-C showed a 6.9 ppm downfield shift in comparison with the non-glycosylated-ara-C. Similarly, the C-5′ signal of ara-C of peak B showed a downfield shift (7.2 ppm). Thus, the structures of peaks A and B were concluded to be 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-β-D-arabinofuranosylcytosine (**1**) and 5-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-β-D-arabinofuranosylcytosine (**2**), respectively (Figure 3).

The glycosylated-ara-Cs, peaks C and D, were converted to **1** and **2**, respectively, by treatment with jack bean β-*N*-acetylhexosaminidase. Therefore, the structures of peaks C and D were determined to be β1-3′- and β1-5′-linked $(GlcNAc)_2$-ara-C (**3**, *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-*O*-β-D-arabinofuranosylcytosine, and **4**, *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→5)-*O*-β-D-arabinofuranosylcytosine) (Figure 3).

Figure 4 shows the time course of the formation of GlcNAc-ara-C and $(GlcNAc)_2$-ara-C. The amount of $(GlcNAc)_2$-ara-C reached a maximum after **1** h, while GlcNAc-ara-C gradually increased and reached a maximum after 6 h. The *B. licheniformis* X-7u chitinase also effected the hydrolysis of **3** to give the hydrolysis products **1** and ara-C (Figure 5). From these results, it is reasonable to assume that $(GlcNAc)_2$-ara-C was produced first followed by the hydrolysis of the nonreducing *N*-acetyl-β-D-glucosamine residue to give GlcNAc-ara-C and finally by the hydrolysis of GlcNAc-ara-C to give GlcNAc and ara-C. The ratio (2:1) of **1**:**2** and **3**:**4** remained fairly constant throughout the reaction. In contrast, **2** was selectively synthesized by the trans-

* To whom correspondence should be addressed.
† Current address: Yamanashi University, Kofu, Yamanashi 400, Japan.
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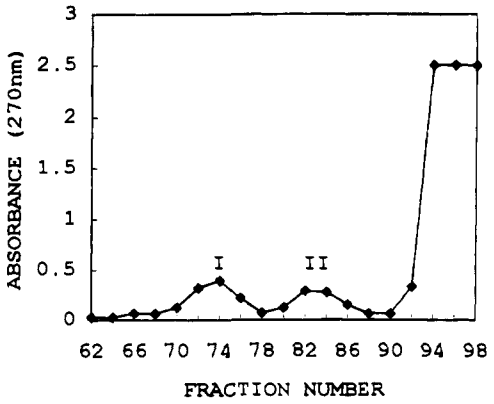

Figure 1. Sephadex G-25 column chromatography of the products of the transglycosylation reaction.

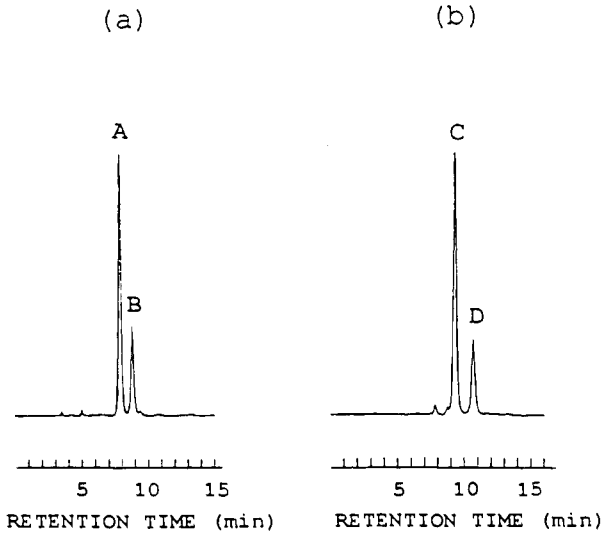

Figure 2. HPLC analyses of peaks I and II in Figure 1: (a) peak I and (b) peak II.

Table 1. ^{13}C NMR Chemical Shifts of 1, **2**, ara-C, and **5**

	1	**2**	ara-C	**5**
C-2	158.0	158.8	158.4	158.2
C-4	167.0	167.1	167.1	167.0
C-5	96.1	96.5	96.2	96.2
C-6	144.0	142.9	143.6	143.8
C-1′	87.2	86.2	86.9	86.9
C-2′	74.8	75.4	76.4	76.2
C-3′	83.3	76.5	76.4	76.3
C-4′	84.5	81.6	84.1	82.7
C-5′	61.3	68.9	61.7	69.4
C-1″	102.2	102.4		104.1
C-2″	56.5	56.5		71.7
C-3″	74.3	74.7		72.2
C-4″	70.8	70.9		69.6
C-5″	76.9	76.9		75.9
C-6″	61.6	61.7		61.8

glycosylation reaction using extracellular β-*N*-acetylglucosaminidase released by cancer cells.

Anticancer Effect of Glycosylated-ara-C. Anticancer activity was measured *in vitro* against ovarian cancer cells (G-401). The cells (1×10^4) were cultured in 200 μL of medium both in the presence and absence of ara-C or $(GlcNAc)_2$-ara-C (**3** + **4**). The number of living cells after 3 days increased over 10 times of those cultured without anticancer reagent (Table 2). In contrast, viable cells could not be detected in the culture in the presence of even 1 μM of ara-C. As can be seen in Table 2, $(GlcNAc)_2$-ara-C (**3** + **4**) depressed the

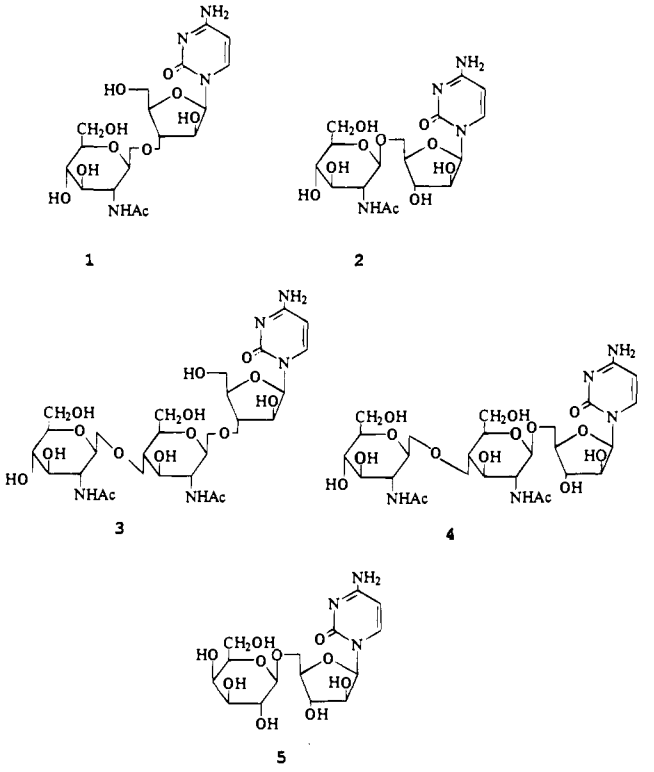

Figure 3. Structure of products **1**–**5** determined by the analyses of ^1H and ^{13}C NMR spectra.

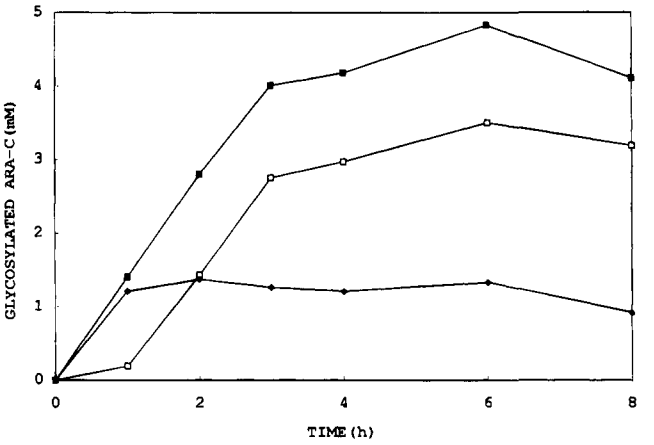

Figure 4. Time courses of the formation of GlcNAc-ara-C (□), $(GlcNAc)_2$-ara-C (◆), and total modified ara-C (■).

growth of G-401 cells when present in a concentration of 1 μM or more. The GlcNAc-ara-C (**1** + **2**) had the same effect (data not shown).

In contrast to the above, 5-*O*-β-D-galactopyranosyl-β-D-arabinofuranosylcytosine (**5**) synthesized by the use of β-galactosidase from *Escherichia coli* indicated no inhibition of G-401 growth. However, when G-401 was cultured in the presence of **5** and β-galactosidase, inhibition of cell growth was observed. This means that while **5** possesses little or no toxicity against G-401 cells, the cytotoxicity against G-401 cells can be made to reappear by hydrolysis of the β-D-galactosyl residue through addition of β-galactosidase. In the case of $(GlcNAc)_2$-ara-C, β-*N*-acetylglucosaminidase secreted from G-401 cells might play the same role as β-galactosidase does in the case of **5**. Consequently, it may be concluded that while GlcNAc-modified ara-C or **5** shows little or no toxicity against G-401 cells when there is no glycosidase, in the presence of their respective

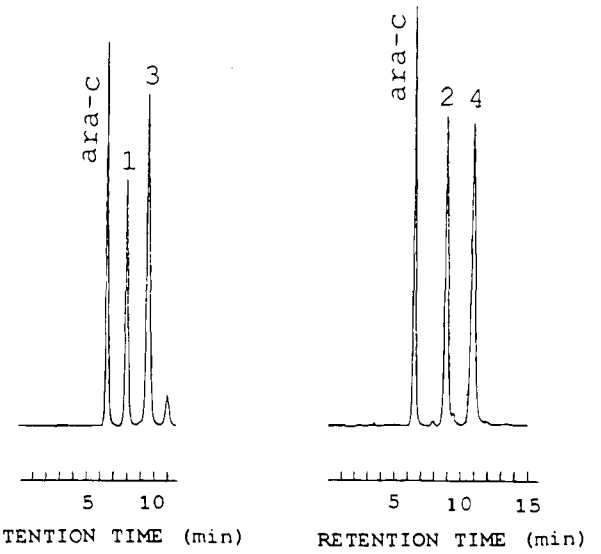

Figure 5. HPLC after the partial digestion of **3** with chitinase.

Table 2. Anticancer Effect of ara-C, **3** + **4**, and **5** against Ovarian Cancer Cells[a]

	cell number ($\times 10^{-4}$)			
dose (μM)	ara-C	**3** + **4**	**5**	**5** + β-galactosidase
0	55	48	55	46
1	0	19	41	15
10	0	7	38	6
100	0	2	56	2

[a] After culture under 5% CO_2 at 37 °C for 3 days, the living cells were counted by trypan blue dye exclusion.

glycosidases, they express cytotoxicity against G-401 cells by virtue of the hydrolysis of their glycosidic bond. Since the linkage between GlcNAc and ara-C of GlcNAc-modified ara-C was hydrolyzed by β-*N*-acetylglucosaminidase released from G-401 cancer cells, only GlcNAc-modified ara-C showed selective toxicity around the cancer cell.

Experimental Section[10]

Human ovarian teratocarcinoma G-401 (CRL 1441) was purchased from ATCC. β-Galactosidase (EC 3.2.1.23) from *E. coli* and β-*N*-acetylhexosaminidase (EC 3.2.1.52) from jack bean were purchased from Toyobo Co. (Osaka, Japan) and Seikagaku Kogyo Co. (Tokyo, Japan), respectively. Nonserum medium CELGROSSER was purchased from Kanto Chemical Co. (Tokyo, Japan). ara-C and 1-*O*-(*p*-nitrophenyl)-2-acetamido-2-deoxy-β-D-glucopyranoside were products of Sigma.

Synthesis of GlcNAc-ara-C (1, 2) and $(GlcNAc)_2$-ara-C (3, 4) by Chitinase from *B. licheniformis* X-7u. A reaction mixture containing 5% $(GlcNAc)_4$, 20% ara-C, and chitinase (0.065 unit/mL) in 20 mM borate buffer (pH 10.0) was incubated at 35 °C for 6 h. After the solution was heated at 100 °C for 5 min, the reaction mixture was loaded onto a Sephadex G-25 column (26 × 900 mm) and eluted with H_2O at a flow rate of 0.25 mL/min. The eluate was monitored by UV adsorption at 270 nm which is characteristic of ara-C. The fractions containing peaks I and II were fractionated by HPLC using an Asahipak NH2P-50 column (4.6 × 250 mm; Showadenko Co.). The glycosylated-ara-C was eluted with an MeCN–water (7:3) mixture. The fractions containing 1–**4** were separated and lyophilized. ^{13}C NMR spectra were obtained in D_2O (internal MeCN, 1.3 ppm) on a Varian XL-400 NMR spectrometer. **1**: 4.2% yield. Anal. ($C_{17}H_{26}N_4O_{10}$) C, H, N. **2**: 2.0% yield. Anal. ($C_{17}H_{26}N_4O_{10}$) C, H, N. **3**: Anal. ($C_{25}H_{39}N_5O_{15}$) C, H, N. **4**: Anal. ($C_{25}H_{39}N_5O_{15}$) C, H, N.

Synthesis of GlcNAc-ara-C (2) by β-*N*-Acetylglucosaminidase from G-401 Cells. The reaction mixture containing 12 mg of 1-*O*-(*p*-nitrophenyl)-2-acetamido-2-deoxy-β-D-glucopyranoside, 120 mg of ara-C, and β-*N*-acetylglucosaminidase (0.4 milliunit/mL) from G-401 cells in 0.1 M sodium acetate buffer (pH 4.0, 500 μL) was incubated at 35 °C for 24 h. After the solution was heated at 100 °C for 5 min, the reaction mixture was analyzed with an Asahipak NH2P-50 column.

Digestion of 3 and 4 with β-*N*-Acetylhexosaminidase from Jack Bean. Either **3** or **4** (25 μg) was dissolved in 20 mM sodium acetate buffer (pH 5.0, 50 μL). Jack bean β-*N*-acetylhexosaminidase (10 milliunits) was added to the glycosylated-ara-C solution. After incubation of the reaction mixture at 35 °C for 6 h, products were analyzed by the same method as used in the synthesis of GlcNAc-ara-C.

Digestion of 3 and 4 with Chitinase. **3** and **4** (50 μg) was dissolved in 40 mM borate buffer (pH 10.0, 100 μL). Chitinase (33 units) was added to the glycosylated-ara-C solution. After incubation of the reaction mixture at 35 °C for 4 h, products were analyzed by the same method as used in the synthesis of **3** and **4**.

Synthesis of 5-*O*-β-D-Galactopyranosyl-β-D-arabinofuranosylcytosine (5). β-Galactosidase (*E. coli*, 341 units) was dissolved in 5 mL of 0.1 M potassium phosphate buffer (pH 8.0) containing 0.45 M lactose and 0.22 M ara-C, and the solution was incubated at 35 °C for 3 h. After heat denaturation of the enzyme, **5** was isolated by the same method as used for GlcNAc-ara-C and $(GlcNAc)_2$-ara-C: 64.7 mg, 15% yield. Anal. ($C_{15}H_{23}N_3O_{10}$) C, H, N.

Anticancer Effect of Glycosylated-ara-C. Human ovarian cancer cells (G-401, 5 × 10^4 cells/mL) were seeded in nonserum medium (CELGROSSER) in the presence or absence of ara-C or glycosylated-ara-C (0–100 μM) and β-galactosidase (*E. coli*, 1 unit/mL). After culture under 5% CO_2 at 37 °C for 3 days, the living cells were counted by trypan blue dye exclusion.

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(10) Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine: Gal-ara-C, 5-*O*-β-D-galactopyranosyl-β-D-arabinofuranosylcytosine; $(GlcNAc)_2$, di-*N*-acetylchitobiose; $(GlcNAc)_4$, tetra-*N*-acetylchitotetraose; $(GlcNAc)_6$, hexa-*N*-acetylchitohexaose.
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