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Oligosaccharide-Linked Acyl Carrier Protein, a Novel Transmethylase Inhibitor, from Porcine Liver Inhibits Cell Growth

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We have previously reported on the identification of the endogenous transmethylation inhibitor oligosaccharide-linked acyl carrier protein (O-ACP). In this study, the role of the transmethylation reaction on cell cycle progression was evaluated using various transmethylase inhibitors, including O-ACP. O-ACP significantly inhibited the growth of various cancer cell lines, including NIH3T3, *ras*-transformed NIH3T3, MDA-MB-231, HT-1376, and AGS. In addition, exposure of *ras*-transformed NIH3T3 to O-ACP caused cell cycle arrest at the G_0/G_1 phase, which led to a decrease in cells at the S phase, as determined by flow cytometry. In contrast, transmethylase inhibitors did not affect the expression of $p21^{WAF1/Cip1}$, a well known inhibitor of cyclin dependent kinase, indicating that the cell cycle arrest by transmethylase inhibitors might be mediated by a $p21^{WAF1/Cip1}$-independent mechanism. Therefore, O-ACP, a novel transmethylase inhibitor, could be a useful tool for elucidating the novel role of methylation in cell proliferation and cell cycle progression.

Key words: *Transmethylation, Cell cycle, Transmethylase inhibitor, Cell proliferation*

INTRODUCTION

Biological methylation is one of the most important ubiquitously occurring reactions, and involves a wide variety of biological compounds, such as nucleic acids, proteins, lipids and amino acids. Protein methylation is classified into N-methylation, O-methylation and S-methylation according to the amino acid residues modified. The side chains of lysine and arginines residues of certain proteins are N-methylated, the free carboxyl groups of glutamyl and aspartyl residues are O-methylated, and the side chains of methionine and cysteine residues are S-methylated (Paik *et al.*, 1986; Paik and Kim, 1990).

The protein arginine methylation reaction by protein arginine methyltransferase 1 (PRMT1) and coactivator-associated protein arginine methyltransferase 1 (CARM1) is associated with the regulation of gene transcription (Chen *et al.*, 1999; Wang *et al.*, 2001). CARM1 and PRMT1, which are responsible for methylation of arginine residues in Histone 3 and Histone 4, respectively, have been demonstrated to be essential for transcriptional activation by nuclear hormone receptors (Chen *et al.*, 1999; Wang *et al.*, 2001). In addition, the protein lysine methylation reaction has been shown to be involved in heterochromatin functions. The methylation of lysine 9 on histone 3 serves as a binding site for the heterochromatin protein, which leads to the formation of heterochromatin (Rice and Allis, 2001). Carboxymethylation of the C-terminal sequence CAAX motif in membrane proteins, such as p21-Ras protein and the related G-protein, was triggered by farnesylcysteine methyltrasferase, leading to the membrane translocation of these proteins (Magee and Marshall, 1999). This process has been considered to a critical step in cell growth and differentiation.

S-Adenosyl-L-methionine (AdoMet) plays a pivotal role as a methyl donor in many biological and biochemical

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events (Chen *et al.,* 1999; Aletta *et al.,* 1998; Paik *et al.,* 1986). The protein methylation reaction is regulated by the balance of the concentrations of AdoMet and an endogenous competitive inhibitor S-Adenosyl-L-homocysteine (AdoHcy) which is produced as a by-product of the AdoMet-dependent transmethylation reaction (Lawrence and Robert-Gero, 1990). AdoHcy hydrolase inhibitors, such as 3-deazaadenosine and 9-(trans-2', trans-3'-dihydroxycyclopent-4-enyl)-adenine, cause intracellular accumulation of AdoHcy, which result in the subsequent inhibition of AdoMet-dependent methyltransferase (Chiang *et al.,* 1992; Liu *et al.,* 1992). The inhibition of the transmethylation reaction leads to the inhibition of cell growth, the induction of cellular differentiation and activation of transcription factors, indicating that the transmethylation reaction might be involved in cell growth and differentiation (Aarbakke *et al.,* 1986; Chiang *et al.,* 1992).

We recently reported on the presence of the endogenous transmethylation inhibitor, oligosaccharide-linked acyl carrier protein (O-ACP) (Seo *et al.,* 2000), which has an inhibitory effect on the S-farnesylcysteine methyltransferase in the p21-Ras signaling pathway (unpublished data).

In this study, we attempted to evaluate the role of the transmethylation reaction on cell cycle progression using various transmethylase inhibitors, including O-ACP. O-ACP significantly inhibited the growth of various cancer cell lines, and caused cell cycle arrest at the G_0/G_1 phase, which led to a decrease in cells at the S phase, as determined by flow cytometry, indicating that the cell cycle progression might be regulated by the transmethylation reaction. However, transmethylase inhibitors did not affect the expression of $p21^{WAF1/Cip1}$, a well known endogenous cell cycle inhibitor, suggesting that the cell cycle arrest by transmethylase inhibitors might be mediated by a $p21^{WAF1/Cip1}$-independent mechanism.

MATERIALS AND METHODS

Cell culture

NIH3T3, *ras*-transformed NIH3T3, MCF-7, MDA-MB-231, HT-1376, and AGS cell lines were maintained in RPMI 1640 or DMEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Growth inhibition assay

Exponentially growing cells were treated with O-ACP, purified from porcine liver, as previously described (Seo *et al.,* 2000), for 24 or 48 h, and the culture medium was removed. The cells were then fixed by incubating with 1 ml of 10% trichloroacetic acid (TCA) at 4°C for 1 h, followed by five washes with distilled water. After complete air-drying of the plate, 0.4% surforhodamine B (SRB) solution in 1% glacial acetic acid was added, at room temperature, for 30 min to stain the cells. Subsequently, the plate was washed five times with 1% glacial acetic acid and allowed to air-dry overnight. Tris-HCl (1 ml, 10 mM) was then added to each well to dissolve the SRB bound to cellular proteins; the SRB absorbance was then measured at 490 nm on an EL 808 ultra microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The SRB absorbance is proportional to the number of cells attached to the culture plate. Therefore, the results of SRB represent the antiproliferative effect of apicidin on mouse and human cancer cell lines.

[^{3}H] Thymidine incorporation assay

DNA synthesis was measured by the levels of incorporation of radioactivity into the DNA from labeled thymidine (Han *et al.,* 2000). Cells were seeded at a density of 5×10^4 per well and incubated in serum-free media for 24 h to synchronize at the G_0 phase. Twenty four hours later, transmethylase inhibitors were added to the cultures, and further incubated for 10 h with 1 μCi of [methyl-^{3}H] thymidine, containing 10% FBS DMEM. After removal of the culture medium, cells were washed twice with 0.5 ml of cold phosphate buffered saline (PBS) trypsinized for 10 min, and transferred to an eppendorf tube. Following the addition of 0.2 ml of 10% TCA and placing in an ice bath for 30 min, the TCA-insoluble materials were collected and solubilized with 0.17 ml of 0.2 M NaOH, containing 0.5% sodium dodecyl sulfate for 30 min at 37°C. The amount of [^{3}H]-labelled thymidine in the whole solutions was counted using a liquid scintillation counter.

Flow cytometry

Flow cytometry was used to analyze the distribution of the DNA contents in the cell population. Following staining with propidium iodide, total fluorescence intensities were determined by quantitative flow cytometry using a BRYTE HS system (Bio-Rad Lab, USA), equipped with a xenon lamp, with an excitation wavelength of 495nm and an emission wavelength of 639nm. Data were analyzed by ModFit (Verity Software House, Inc, Topsaham, ME).

Transfection and luciferase assay

The human wild-type $p21^{WAF1/Cip1}$ promoter-luciferase fusion plasmid, pWWP, was kindly gifted by Dr. Yoshihiro Sowa (Kyoto Prefectural University of Medicine). HeLa cells were plated into 6-well plates at a density of 1×10^5

per well, and incubated for 24 h. For the analysis of the $p21^{WAF1/Cip1}$ promoter, cells were transfected with 5 μg per well of the $p21^{WAF1/Cip1}$ promoter reporter plasmid DNA using the ProFection transfection reagent (Promega, Madison, WI). Following 24 h of transfection, the medium was changed for medium with or without each inhibitor, and the cell lysates were collected for the luciferase assay 24 h later. The luciferase activities of the cell lysates were measured according to the manufacturers recommendations (Promega, Madison, WI).

Immunoblotting

HeLa cell lysates were boiled in Laemmli sample buffer for 3 min, and 30 μg of each total protein subjected to SDS-polyacrylamide gel electrophoresis (PAGE), on 15% slab gels for the analysis of the $p21^{WAF1/Cip1}$. Proteins were transferred to polyvinylidene difluoride membranes, which were blocked for 30 min in tris-buffered saline (TBS) containing: 0.1% tween 20, and 5% (w/v) dry skim milk powder, and incubated overnight with anti-$p21^{WAF1/Cip1}$ (Santa Cruz Biotechnologies, Inc.). The membranes were then washed with TBS containing 0.1% tween 20 and incubated for 1 h with an anti-rabbit secondary antibody. Bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (Bio-Rad).

RESULTS AND DISCUSSION

Antiproliferative activities of the transmethylase inhibitors on the growth of various cancer cell lines

AdoHcy, a well-known transmethylase inhibitor, is known to inhibit AdoMet-dependent methyltransferases in a competitive manner, and cell growth at micromolar concentrations (Ueland, 1982). We first determined the effect of the newly identified transmethylase inhibitor, O-ACP on the proliferation of various cancer cell lines. Treatment of NIH3T3, *ras*-transformed NIH3T3, MDA-MB-231, HT-1376 and AGS cell lines with AdoHcy (0.5 mM) and O-ACP (2 mM) inhibited the growth of the various cancer cells by more than 50%. These inhibitors exhibited different susceptibility to the cell lines: The inhibitory potency of O-ACP in MDA-MB-231 and AGS cell lines being stronger than that of AdoHcy (Table I).

To further confirm their inhibitory effects on the cell growth, the DNA syntheses were measured following treatment of NIH3T3 and *ras*-transformed NIH3T3 cells with various concentrations of the transmehylase inhibitors using a [^{3}H]-thymidine incorporation assay. AdoHcy almost completely inhibited the [^{3}H]-thymidine incorporation to the serum-starved control level, at a concentration of 1 mM, in *ras*-transformed NIH3T3 cell, but not in NIH3T3 cells, whereas O-ACP markedly ablated the [^{3}H]-thymidine incorporation in both cell lines at a concentration of 4 mM (Fig. 1).

Table I. Effect of transmethylase inhibitors on the growth of various cell lines*.

Cell line	AdoHcy	O-ACP	Cell No. (% of control)	
			Day 2	Day 4
NIH3T3	+	-	36.33 ± 8.05	17.75 ± 0.63
	-	+	23.32 ± 4.36	16.11 ± 2.90
ras-transformed NIH3T3	+	-	44.35 ± 16.87	51.33 ± 5.76
	-	+	50.75 ± 17.26	57.16 ± 7.70
MCF-7	+	-	77.50 ± 15.09	67.79 ± 13.64
	-	+	63.92 ± 11.21	65.39 ± 21.75
MDA-MB-231	+	-	81.43 ± 0.31	51.77 ± 2.20
	-	+	26.54 ± 0.84	10.97 ± 9.08
HT-1376	+	-	97.38 ± 3.89	63.10 ± 6.23
	-	+	77.81 ± 2.28	53.53 ± 4.46
AGS	+	-	89.68 ± 1.56	10.88 ± 0.53
	-	+	43.33 ± 4.36	10.64 ± 2.70

*AdoHcy and O-ACP were treated with 0.5 mM and 2 mM in various cell lines, respectively. After incubation for 2 or 4 days, the cell number was counted as described in "Material and Methods". Data shown represent relative cell numbers in cells treated with trenamethylase inhibitor as a percentage of the cell numbers in control (mean ± s.d.).

Table II. Effects of transmethylase inhibitors on cell cycle progression*

Cells	Cell cycle	Control	Serum	AdoHcy		O-ACP	
				0.5 mM	1 mM	1 mM	2 mM
NIH3T3	G_0/G_1	76.2	10.9	42.9	49.1	56.5	80.3
	S	9.5	51.5	27.7	25.0	19.8	7.9
	G_2/M	14.3	37.6	39.4	35.9	23.7	11.8
ras-transformed NIH3T3	G_0/G_1	44.9	27.3	43.3	53.3	43.2	48.9
	S	29.1	47.7	15.4	13.1	25.5	21.6
	G_2/M	26.1	25.0	41.4	33.7	31.3	29.5

*Serum-starved NIH3T3 and *ras*-transformed NIT3T3 cells were cultured in DMEM containing 10% FBS with or without the indicated concentrations of transmethylase inhibitors. After 22 h incubation, cells were collected and analyzed by Flow Cytometer.

Effect of transmethylase inhibitors on the cell cycle progression

Since both cell growth and DNA synthesis were inhibited considerably by the O-APC, we examined its effect on the cell cycle progression using flow cytometry. As shown in Table II, an increase in cells at the S phase induced by serum treatment (51.5%) was dramatically inhibited by pretreatment with the transmethylase inhibitors, AdoHcy (1 mM) and O-ACP (2 mM), up to 25% and 7.9%, respectively. A similar effect was observed in

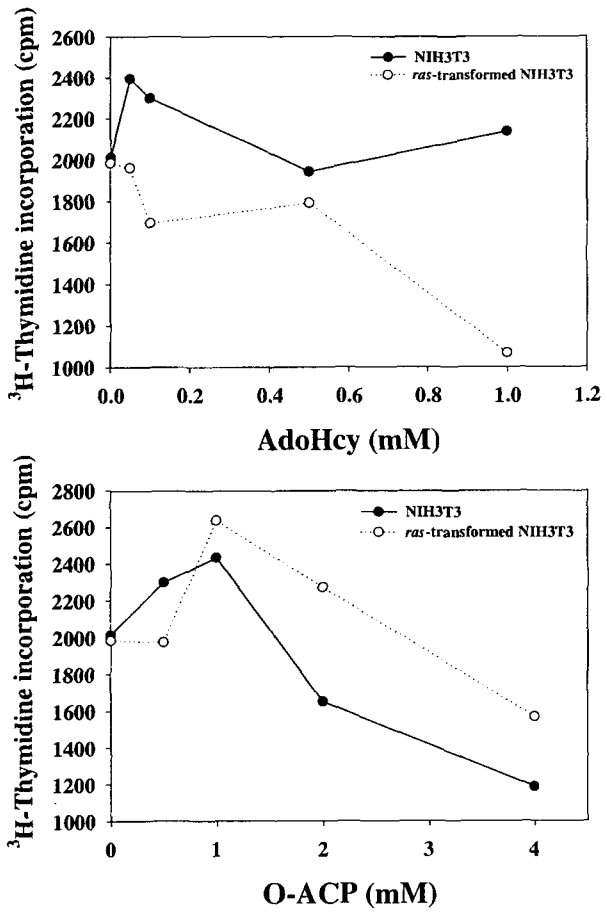

Fig. 1. The transmethylase inhibitors, AdoHcy and oligosaccharide-linked ACP, inhibit the proliferation of NIH3T3 and *ras*-transformed NIH3T3 cells. NIH3T3 and ras-transformed NIH3T3 cells (seeded at a density of 5×10^4 cells/well in 6 well dishes) were treated with various concentrations of transmethylase inhibitors. Data shown are expressed as counts per minutes.

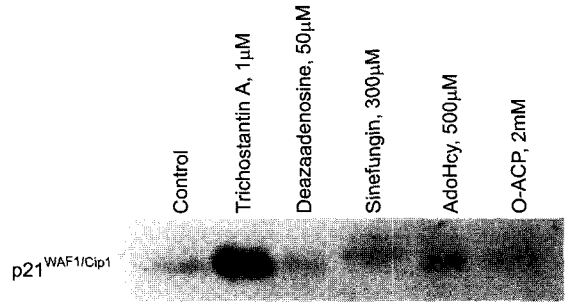

Fig. 2. Effect of various transmethylase inhibitors on the p21$^{WAF1/Cip1}$ expression. HeLa cells were cultured in DMEM containing 10% FBS. After treatment with indicated inhibitors for 24 h, the cells lysed and analyzed with immunoblotting using specific antibody for p21$^{WAF1/Cip1}$.

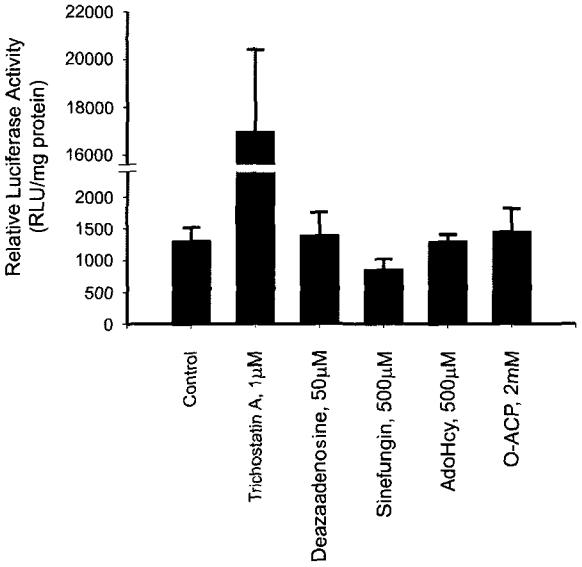

Fig. 3. Effect of various transmethylase inhibitors on the p21$^{WAF1/Cip1}$ promoter activity. HeLa cells were cultured in DMEM containing 10% FBS. pWWP, the full promoter of p21$^{WAF1/Cip1}$, was transiently transfected into HeLa cells using the calcium phosphate method, and luciferase activities were measured after incubation with or without the indicated concentrations of various inhibitors for 24 hr. Relative luciferase activity is shown as raw lights units (RLU) in cell lysates/1 mg protein. The data are shown as the means (bars, S.E.) (n=3).

ras-transformed NIH3T3 cells. These results correlated well with those of the inhibitory effects on the cell growth (Table I) and thymidine incorporation assay (Fig. 1), indicating that inhibition of the cell growth by transmethylase inhibitors might be due to arrest of the cell cycle progression at the G_0/G_1 phase.

Effect of transmethylase inhibitors on the expression of p21$^{WAF1/Cip1}$

Cell cycle progression from the G_0/G_1 to the S phase is regulated by the sequential activation of cyclin-dependent kinases (Cdks), whose activation is carefully regulated at multiple levels, including the induction and degradation of cyclin protein, Cdk phosphorylation by cyclin-activating kinase, and the induction of Cdk inhibitory proteins (Draetta, 1994; Hunter and Pines, 1994; King *et al.*, 1994; Sherr, 1994; Scherr and Roberts, 1995). Cdk inhibitor, which include p21$^{WAF1/Cip1}$, p27^{Kip1}, p57^{Kip2}, p16, p15, p18 dand p19, can negatively regulate cell cycle progression by inhibiting the Cdk activity through the physical association with their target cyclin-Cdk complexes. p21$^{WAF1/Cip1}$ was first cloned and characterized as an important effector that acts to block the cyclin E-Cdk2 complex kinase activity in the p53-mediated cell cycle arrest induced by DNA damage (Dulic *et al.*, 1994; El-Deiry *et al.*, 1993; El-Deiry *et al.*, 1994; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1992; Xiong *et al.*, 1993; Waga *et al.*, 1994). We thus attempted to examine if the cell cycle arrest at the G_0/G_1 phase by transmethylase inhibitors was attributed to the induction of p21$^{WAF1/Cip1}$ expression. The p21$^{WAF1/Cip1}$ expression was dramatically

increased by treatment with trichostatin A, a well known histone deacetylase inhibitors (Yoshida *et al.,* 1990). It has been repeatedly demonstrated that trichostatin A increased the $p21^{WAF1/Cip1}$ expression in various cell types, leading to the inhibition of the cell cycle progression from the G_1 to the S phase (Siavoshian *et al.,* 2000; Sowa *et al.,* 1999). However, treatment with various transmethylase inhibitors, including O-ACP, AdoHcy, sinefungin and deazaadenosine, showed no alteration to the $p21^{WAF1/Cip1}$ expression (Fig. 2). We further confirmed the effects of various transmethylase inhibitors on the promoter activity of the $p21^{WAF1/Cip1}$ gene using a reporter gene assay. As shown in Fig. 3, the promoter activity was increased 13-fold by treatment with trichostatin A. However, the transmethylase inhibitors did not alter the promoter activity of the $p21^{WAF1/Cip1}$, which was accompanied with no induction of the protein expression. These data indicate that the cell cycle arrest by transmethylase inhibitors might be attributed to another mechanism rather than the induction of the $p21^{WAF1/Cip1}$.

In summary, O-ACP significantly inhibited the growth of various cancer cell lines, including NIH3T3, *ras*-transformed NIH3T3, MDA-MB-231, HT-1376 and AGS. Its inhibitory effects on cell growth might be due to the inhibition of DNA synthesis through the arrest of the cell cycle at the G_0/G_1 phase, which was independent of the $p21^{WAF1/Cip1}$ protein expression. Taken together, these results suggest that cell growth may be regulated by transmethylation reactions through an independent mechanism of the $p21^{WAF1/Cip1}$. Therefore, we are in the process of trying to clarify the mechanism of action of O-ACP on the inhibition of cell growth and cell cycle progression.

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