

W. J. LEE AND B. T. ZHU [2005] MED HYPOTHESES RES 2: 325-337.

## MODULATION OF THE RATE OF ENZYMATIC DNA METHYLATION BY CATECHOL-O-METHYLTRANSFERASE

WON JUN LEE AND BAO TING ZHU\*

DEPARTMENT OF BASIC PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY, UNIVERSITY OF SOUTH CAROLINA, COLUMBIA, SC 29208, USA

**ABSTRACT.** WE EXAMINED THE MODULATING EFFECTS of catechol-O-methyltransferase (COMT) and *S*-adenosyl-*L*-homocysteine (AdoHcy) on DNA methylation catalyzed by prokaryotic SssI DNMT and human DNMT1. The presence of COMT (at physiologically-relevant concentrations) enhanced the rate of DNA methylation catalyzed by SssI DNMT and human DNMT1, by up to 1-fold over the control rate. Transfections of the human COMT siRNAs into the cultured MCF-7 human breast cancer cells for 10 days caused ~50% reduction of the COMT activity, and this reduction in COMT was accompanied by a slight decrease in the methylation status of the RAR $\beta$  gene. Kinetic analyses showed that AdoHcy strongly and noncompetitively inhibited the methylation of DNA by competing *S*-adenosyl-*L*-methionine off the DNMT, thus shifting more enzyme molecules to a form that was bound with AdoHcy. Consequently, the  $V_{MAX}$  values were reduced when AdoHcy was present, but the  $K_M$  values were not markedly altered. As expected, the presence of COMT increased the  $V_{MAX}$  for the enzymatic DNA methylation, but the  $K_M$  values were essentially not changed. In conclusion, the COMT-mediated enhancement of DNA methylation likely is due to the sequestration of AdoHcy by binding to COMT, which reduces the intracellular bioavailability of the free AdoHcy for DNMT inhibition.

\*ADDRESS ALL CORRESPONDENCE TO: DR. BAO TING ZHU, DEPARTMENT OF BASIC PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY, UNIVERSITY OF SOUTH CAROLINA, ROOM 617 OF COKER LIFE SCIENCES BUILDING, 700 SUMTER STREET, COLUMBIA, SC 29208 (USA).

PHONE: 803-777-4802; FAX: 803-777-8356; E-MAIL: BTZhu@cop.sc.edu

## 1. INTRODUCTION

Studies have shown that DNA methylation at the C-5 position of cytosine within the CpG dinucleotides represents an important mechanism for epigenetic control of gene expression and maintenance of genome integrity. While DNA hypermethylation is associated with inactivation of genes, global genomic hypomethylation is often associated with the induction of chromosomal instability [1]. DNA methylation has been shown to play a central role in gene imprinting [2], embryonic development [3,4], X-chromosome gene silencing [5], and cell cycle regulation [6]. Moreover, recent studies have shown that global hypomethylation, accompanied by region-specific hypermethylation, is a common characteristic in many tumor cells [7-9], and it is suggested that aberrant DNA methylation is among the earliest changes during oncogenesis [10].

DNA methylation is catalyzed by specific DNA methyltransferases (DNMTs). Multiple DNMTs are present in humans, animals, and microorganisms, and they have varying degrees of specificity towards the unmethylated and hemi-methylated DNA substrates [11]. Like other enzymatic methylations, such as the catechol-O-methyltransferase (COMT)-mediated O-methylation of various catechol substrates, the DNMT-mediated methylation of DNA also uses *S*-adenosyl-*L*-methionine (AdoMet) as the methyl donor, resulting in the formation of *S*-adenosyl-*L*-homocysteine (AdoHcy) after donating its methyl group to the DNA substrate. A number of earlier studies have indicated that AdoHcy may serve as a universal feedback inhibitor for various AdoMet-dependent methyltransferases [12-15]. In the present study, we have explored the possible modulating effects of COMT and AdoHcy on the rate of enzymatic DNA methylation *in vitro*. The prokaryotic SssI DNMT (M.SssI) and human DNMT1 have been employed as tools in our present study because these two DNMTs have differential preference for unmethylated and hemi-methylated DNA substrates. While the prokaryotic SssI DNMT is functionally similar to the human DNMT3A and 3B and methylates both unmethylated and hemi-methylated DNA substrates with almost equal efficiency [16], the human DNMT1 preferentially methylates hemi-methylated DNA substrate. In order to probe whether alterations of the levels of

COMT in a cell could alter the status of DNA methylation, we used the human COMT siRNAs to probe their effects on the global DNA methylation status and also on the methylation status of a representative gene (retinoic acid receptor, RAR $\beta$ ) in the cultured MCF-7 cells. The MCF-7 cells were used in this study because our preliminary assays showed that this cell line expressed higher activity of the COMT than a few other human breast cancer cell lines (such as MDA-MB-231 and MDA-MB-435).

## 2. MATERIALS AND METHODS

### 2.1. CHEMICALS AND REAGENTS

The synthetic double-stranded poly(dG-dC)·poly(dG-dC) and poly(dI-dC)·poly(dI-dC) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The average length of the poly(dG-dC)·poly(dG-dC) and poly(dI-dC)·poly(dI-dC) was 920 and 5469 base pairs, respectively. The SssI DNMT (M.SssI) and human DNMT1 were obtained from the New England Biolabs (Beverly, MA). During the course of the study, multiple lots of the SssI DNMT and human DNMT1 enzymes from the same supplier were used. According to the supplier, the SssI DNMT was isolated from a strain of *E. coli* transfected with the SssI DNMT gene from the *Spiroplasma* sp. strain MQ1 (16), and it was stored at  $-20^{\circ}\text{C}$  (at 200 units/50  $\mu\text{L}$ ) in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 200  $\mu\text{g}/\text{ml}$  BSA, and 50% glycerol. DNMT1 was stored in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 7  $\mu\text{g}/\text{ml}$  PMSF, and 50% glycerol, and the protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E64, bestatin, leupeptin and aprotinin. It should be noted that different batches of the enzymes gave quite different overall catalytic activities and  $V_{MAX}$  values (up to 5-fold), but the  $K_M$  values of the enzymes appeared to be quite consistent. A serum-free Opti-MEM I medium, AdoMet, AdoHcy, and porcine liver COMT (affinity column-purified, 1396 units/mg of protein) were purchased from the Sigma Chemical Co. (St. Louis, MO). [Methyl- $^3\text{H}$ ]AdoMet (specific activity of 11.2-13.5 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, MA).

The scintillation cocktail (ScintiVerse BD) was obtained from Fisher Scientific (Pittsburgh, PA).

## 2.2. ASSAY OF DNA METHYLATION IN VITRO

The following reaction mixtures were freshly prepared on ice immediately prior to measuring the *in vitro* DNA methylation. The poly(dG-dC)-poly(dG-dC) and poly(dI-dC)-poly(dI-dC) substrates were diluted in the TE buffer (containing 10 mM Tris-HCl and 1 mM EDTA, pH 7.6) to desired stock concentrations, usually at 250  $\mu$ M of the CpG methylation sites. Note that 1 mole of the double-stranded dG-dC:dG-dC and dI-dC:dI-dC dinucleotides contains 2 moles of the CpG methylation sites. The stock solutions of SssI DNMT and human DNMT1 were further diluted usually to a concentration of 1 unit/5  $\mu$ L. The methyl donor AdoMet (containing  $\sim$ 0.5  $\mu$ Ci [methyl- $^3$ H]AdoMet) was dissolved in 0.87 mM HEPES at desired stock concentrations depending on the experiments. As recommended by the supplier of the DNMTs, the reaction buffer for SssI DNMT consisted of 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (pH 7.9), and the reaction buffer for DNMT1 consisted of 50 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol (pH 7.8).

The above freshly-prepared solutions were then added in a sequential order to a 1.5-mL microcentrifuge tubes on ice: 5  $\mu$ L of the reaction buffer, 5  $\mu$ L of the synthetic DNA substrate, 5  $\mu$ L of the enzyme, 5  $\mu$ L of water, and 5  $\mu$ L of AdoMet (mixed with [methyl- $^3$ H]AdoMet). As such, the final reaction mixture usually contained 0.125–10  $\mu$ M of CpG methylation sites, 1.25–20  $\mu$ M of AdoMet (containing  $\sim$ 0.5  $\mu$ Ci [methyl- $^3$ H]AdoMet), and 0.25–4 units of the enzyme in a final volume of 25  $\mu$ L. Notably, in many of the experiments designed to study the effects of AdoHcy and COMT, the 5- $\mu$ L volume of water was replaced by AdoHcy and/or COMT. Considering that since Mg<sup>2+</sup> ion is a cofactor needed for the activity of COMT, 2 mM of MgCl<sub>2</sub> was added into DNMT1-mediated reaction tube because the corresponding reaction buffer provided by the supplier did not contain MgCl<sub>2</sub>. The incubations were carried out at 37°C for varying lengths of time, and the reactions were arrested by immediately placing the tubes to ice-cold

temperatures, followed by addition of 145  $\mu$ L ice-cold 0.9% sodium chloride and 100  $\mu$ L salmon testes DNA (at 1 mg/mL in the TE buffer). To precipitate the DNA, 30  $\mu$ L of 3 M sodium acetate and 900  $\mu$ L ice-cold ethanol (95%) were added, and the samples were placed in a  $-80^\circ\text{C}$  freezer for 2 hrs. After centrifugation at  $\sim$ 10,000 g for 10 min, 100  $\mu$ L of the heat-deactivated flour (suspended at 40 mg/ml in double-distilled water) was added to each tube, followed by centrifugation at  $\sim$ 10,000 g for 5 min. This step was designed to firmly secure the precipitated DNA pellet (including the methylated poly[dG-dC]-poly[dG-dC] or poly[dI-dC]-poly[dI-dC]) at the bottom of the microcentrifuge tubes which would prevent the pellet from being partially washed away during the following washing steps. Notably, our comparison of various measurements showed that the addition of the flour made the intra-assay as well as inter-assay variations much smaller when compared to the parallel assays without flour. The pellets were then gently washed 3 times with 70% ethanol, and each wash was followed by centrifugation for 3 min at 10,000 g. The pellets (containing DNA and flour) were then resuspended in 30  $\mu$ L of 100% ethanol and 1 mL of the TE buffer (pH 7.6). Each vial was sonicated for  $\sim$ 10 min and vortexed thoroughly to assure adequate resuspension of the pellet, and then the content was transferred to a scintillation vial (containing 4 mL of ScintiVerse BD) for measurement of  $^3$ H-radioactivity with a liquid scintillation counter (Packard Tri-CARB 2900 TR; Downers Grove, IL). Blank values obtained from incubations in the absence of the DNA substrate were also determined in each individual assay and subtracted. Since the same amount (1 unit) of the SssI DNMT and DNMT1 was used in most of the assays (unless otherwise indicated), the rate of DNA methylation, mediated by SssI DNMT and DNMT1, was thus expressed as “PMOL OF METHYLATED PRODUCTS FORMED/MIN” (“PMOL/MIN”). The kinetic parameters ( $K_M$  and  $V_{MAX}$  values) were calculated manually according to the Eadie-Hofstee plots ( $V$  vs  $V/[S]$ ).

## 2.3. DETERMINATION OF THE METHYLATION STATUS OF RAR $\beta$ GENE IN MCF-7 CELLS

The human MCF-7 breast cancer cell line used in this study was obtained from the American Type

Culture Collection (Manassas, VA), and its culture conditions were described in our recent study [17]. We used the specifically-designed siRNAs for the human COMT gene to suppress the expression of COMT in MCF-7 cells. The target sequences of the COMT siRNAs were designed according to the human COMT sequence (GenBank accession number NM\_001970) and the design guidelines suggested by Ambion (Austin, TX). The sense sequence of the COMT siRNA was GGCUCAUCACCAUCGAGAUTT, and the antisense sequence was AUCUCGAUGGUGAUGAGCCTC. The siRNAs for human COMT were annealed by incubation at 90°C for 1 min, followed by incubation at 37°C for 1 hr. Twenty-four hr before transfection, the MCF-7 cells were plated into 6-well culture plate at 0.5 million cells per well in the regular growth medium (EMEM, 10% FBS) and incubated overnight under regular culture conditions. Transfection medium contained 50 nM or 100 nM COMT siRNAs in a serum-free Opti-MEM I medium. To initiate the

transfection, the cells were washed once with Opti-MEM I medium, and 800  $\mu$ L of Opti-MEM I medium was then added to each well and followed by addition of 200  $\mu$ L of the transfection agent-siRNAs mixture. The cells were incubated in the incubator for 4 hr. After this incubation, 3 mL of medium containing 10% FBS was added to each well, and the cells were incubated for 72 hr before the second transfection. The cells were transfected 3 times before they were harvested for preparation of cell homogenates and DNA. To determine the effect of COMT siRNAs on the expression of COMT, we determined COMT enzymatic activity for the methylation of quercetin according to the method described previously (14, 18). Briefly, the reaction mixture consisted of cell lysates, 2 mM MgCl<sub>2</sub>, 20  $\mu$ M AdoMet (~1  $\mu$ Ci [methyl-<sup>3</sup>H]AdoMet), 1 mM dithiothreitol, and 5  $\mu$ M quercetin (as substrate) in 200  $\mu$ L Tris-HCl buffer (10 mM, pH 7.4). The reaction was initiated by addition of cell lysates and carried out at 37°C for 30 min, and the reaction

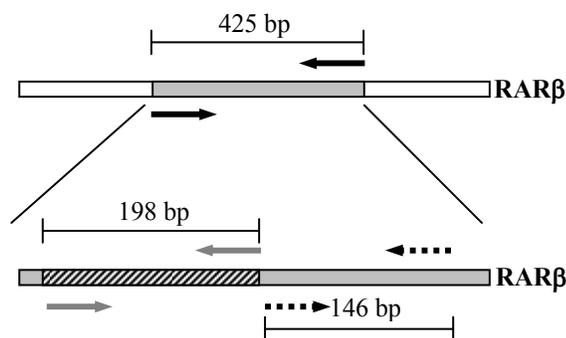
TABLE 1. STRATEGIES FOR THE 2-STAGE NESTED PCR.

Gene		Forward Primers (5'-3')	Reverse Primers (5'-3')	Annealing temperature (°C)	Product size (bp)
RAR $\beta$	<i>N</i> :	TTAAGTTTGTGAGAATTTG	CCTATAATTAATCCAAATAATCATTACC	60	425
	<i>IC</i> :	AGATTAGTTGGGTTATTTGAA	CATCCCAATCCTCAAACAA	53	198
	<i>U</i> :	TTGAGAATGTGAGTGATTGA	AACCAATCCAACCAAAACAA	67	146
	<i>M</i> :	TCGAGAACGCGAGCGATTTCG	GACCAATCCAACCGAAACGA	70	146

*N*, nest primers; *IC*, internal control primers; *U*, unmethylation-specific primers; and *M*, methylation-specific primers

Stage-I PCR

Stage-II PCR



Nest primers	Stage-I PCR product
Internal control primers	Stage-II PCR product
Unmethylation and Methylation-specific primers	

mixture was then extracted with 5 mL of ethyl acetate. The organic extracts were measured for radioactivity content with a liquid scintillation counter.

To determine the DNA methylation status in MCF-7 cells, DNA was extracted using the DNeasy tissue kit (Qiagen, Valencia, CA). The global methylation status as determined by using the reverse-phase HPLC method as described earlier [19]. The methylation status of RAR $\beta$  gene was determined by using a nested 2-stage PCR approach [20]. First, the extracted DNA (1  $\mu$ g) was modified by the EZ DNA methylation kit (Zymo Research, Orange, CA) under conditions specified by the supplier. The unmethylated cytosine was then converted to uracil, whereas 5-methylcytosine remained unaltered. Sodium bisulfite-modified DNAs were then subjected to the STAGE-I PCR to amplify the 425-bp fragments of the RAR $\beta$  gene (see TABLE 1). The STAGE-I PCR products include a portion of the CpG-rich promoter region of each gene. The primers (from Invitrogen Life Technologies, Frederick, MD) for STAGE-I PCR recognized the bisulfite-modified template but did not discriminate between methylated and unmethylated alleles. The STAGE-I PCR products were diluted 50 to 1000 times, and 2.5  $\mu$ L of the diluted PCR products was subjected to a STAGE-II PCR with primers specifically designed for the methylated or unmethylated templates. The sequences of the primers, annealing temperatures, and product sizes used in the STAGE-I and STAGE-II PCR amplifications of the RAR $\beta$  gene are summarized in TABLE 1. HotStarTaq DNA polymerase (Qiagen, Valencia, CA) in a 25- $\mu$ L reaction volume was used in all PCRs. The conditions for the STAGE-I PCR were as follows: 95°C for 15 min, then denaturing at 95°C for 45 s, annealing at 60°C for 1 min, extension at 72°C for 1 min for 40 cycles followed by a final extension at 72°C for 10 min. Conditions for the STAGE-II PCR included 95°C for 15 min, then denaturing at 95°C for 45 s, annealing at 67°C (unmethylation-specific) and 70°C (methylation-specific) for 45 s, extension at 72°C for 45 s for 40 cycles and a final 10-min extension step at 72°C. The number of cycles and the PCR conditions were optimized so that the amplified signal was still on the linear portion of the amplification curve.

Controls (without DNA) were included for each set of PCRs. Amplified PCR products were subjected to electrophoresis using 2% agarose gels, stained with ethidium bromide, and directly visualized with ultraviolet transillumination, and photographed. The integrated optical density (IOD) of each band was quantified by densitometry. The relative levels of methylated and unmethylated PCR products were normalized against the PCR products obtained by using another set of primers specifically designed to amplify the regions which served as internal standard for the STAGE-I PCR products (depicted in TABLE 1). The primers for the internal standard would not discriminate between methylated and unmethylated alleles. All the PCR amplifications were carried out using a PTC-100 thermal cycler (MJ Research, Waltham, MA).

### 3. RESULTS

#### 3.1. MODULATION OF SSSI DNMT-MEDIATED DNA METHYLATION BY COMT

Before we characterized the modulating effects of COMT and AdoHcy on enzymatic DNA methylation, we first optimized the reaction conditions for the *in vitro* DNA methylation by determining the effects of the incubation time and the effects of different concentrations of each DNMT (SssI DNMT and human DNMT1), of the methyl donor AdoMet, and of the synthetic DNA substrates (DATA NOT SHOWN). Based on our measurements, a common reaction condition was found to be suitable for both SssI DNMT- and DNMT1-mediated DNA methylation, which included an incubation time of 30 min, an enzyme concentration of 1 unit/25  $\mu$ L, an AdoMet concentration of 5  $\mu$ M, and the substrate concentrations from 0.1–5  $\mu$ M.

To determine the potential modulating effects of the COMT system on SssI DNMT-mediated DNA methylation, varying amounts of the porcine liver COMT (0.5–8 units, i.e., 0.36–5.73  $\mu$ g) were added to the incubation mixture (final volume of 25  $\mu$ L). As shown in FIG. 1A, the presence of COMT caused a concentration-dependent increase in the rate of SssI DNMT-mediated methylation of the poly(dG-dC)-poly(dG-dC) substrate, and the maximum enhance-

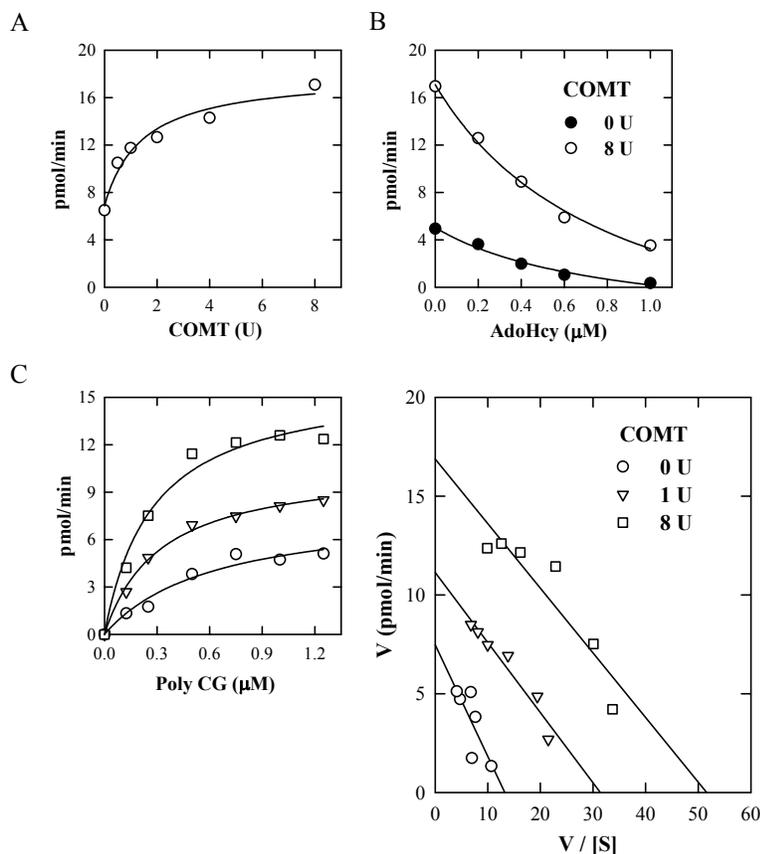


FIGURE 1. THE EFFECTS OF COMT ON THE KINETICS OF THE SssI DNMT-MEDIATED DNA METHYLATION IN VITRO. A: Concentration dependence for the enhancement of DNA methylation by COMT. The incubation mixture consisted of 1  $\mu$ M poly(dG-dC)-poly(dG-dC), 5  $\mu$ M AdoMet (containing  $\sim 0.5$   $\mu$ Ci [methyl- $^3$ H]AdoMet), 1 unit of SssI DNMT, and 0.5-8 units of COMT in a final volume of 25  $\mu$ L. B: Inhibition of the SssI DNMT-mediated DNA methylation by AdoHcy in the absence or presence of COMT. AdoHcy concentrations used were 0.2, 0.4, 0.6, and 1.0  $\mu$ M. C: The left panel illustrates the rate of SssI DNMT-catalyzed DNA methylation in the absence or presence of AdoHcy, and the right panel shows the Eadie-Hofstee plot of the same data set. The concentrations of poly(dG-dC)-poly(dG-dC) were 0.125, 0.25, 0.5, 0.75, 1.0, and 1.25  $\mu$ M. Incubations were carried out at 37°C for 30 min in the presence of MgCl<sub>2</sub> (10 mM). The batch number of SssI DNMT used for generating the data in A, B, C was 16-10, and that for the data in D was 16-3. Each data point was derived from at least two replicate measurements.

ment was  $\sim 1$ -fold over the control values when 8 units (5.73  $\mu$ g) of the COMT were present. It should be noted that the concentrations of porcine liver COMT used in the present study were considered to be physiologically-relevant because our measurements showed that COMT at these concentrations provided only a moderate rate of methylation of representative catechol substrates (such as catechol estrogens and tea catechins) in vitro (DATA NOT SHOWN). Also, the observed enhancing effect of COMT was not due to a nonspecific stabilizing effect exerted by the presence of the COMT protein, because the presence of BSA at up to 1 mg/ml did

not have any detectable enhancing effect on the rate of enzymatic DNA methylation in vitro (DATA NOT SHOWN).

In additional experiments, we found that the presence of COMT slightly decreased the inhibitory potency and efficacy of AdoHcy on DNA methylation (FIG. 1B). Kinetic analysis with two different lots of the SssI DNMT showed that the presence of COMT at 1 and/or 8 units increased the  $V_{MAX}$  values of the DNA methylation in a concentration-dependent manner, but it had little effect on the  $K_M$  values (FIG. 1C, 1D), which was as expected. These data suggested that the enhancing

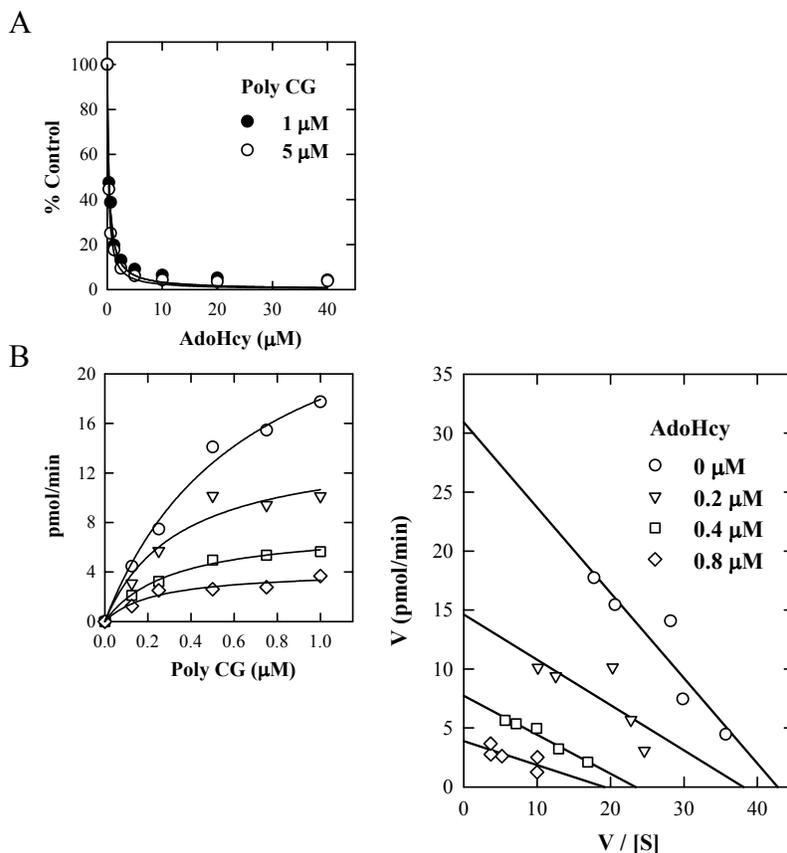


FIGURE 2. THE EFFECT OF ADOHCY ON THE SSSI DNMT-MEDIATED DNA METHYLATION. A: Substrate concentration independence of the inhibitory effect of AdoHcy on DNA methylation. The incubation mixture consisted of 1 or 5  $\mu\text{M}$  poly(dG-dC)-poly(dG-dC), 5  $\mu\text{M}$  AdoMet (containing 1.0  $\mu\text{Ci}$  [methyl- $^3\text{H}$ ]AdoMet), 0–40  $\mu\text{M}$  AdoHcy, and 1 unit of SssI DNMT (lot No. 16-10) in a final volume of 25  $\mu\text{L}$ . B: The left panel illustrates the rate of SssI DNMT-catalyzed DNA methylation in the absence or presence of AdoHcy, and the right panel shows the Eadie-Hofstee plot of the same data set. The reaction conditions consisted of 0.15–1.0  $\mu\text{M}$  poly(dG-dC)-poly(dG-dC), 5  $\mu\text{M}$  AdoMet (containing 0.5  $\mu\text{Ci}$  [methyl- $^3\text{H}$ ]AdoMet), 0–0.8  $\mu\text{M}$  AdoHcy, and 1 unit of SssI DNMT in a final volume of 25  $\mu\text{L}$ . Incubations were carried out at 37°C for 30 min in the presence of 10 mM  $\text{MgCl}_2$ . Each data point was derived from at least two replicate measurements.

effect of COMT on SssI DNMT-mediated DNA methylation likely was due to the binding of AdoHcy with COMT and thereby decreasing the free concentrations of AdoHcy available for inhibition of the SssI DNMT.

To further understand the mechanism(s) for the observed enhancement of DNA methylation by COMT, we studied the modulating effects of AdoHcy on SssI DNMT-mediated DNA methylation. We found that AdoHcy was a very potent inhibitor of SssI DNMT-mediated DNA methylation in vitro, and the estimated  $IC_{50}$  values for AdoHcy were  $\sim 0.25$   $\mu\text{M}$ , regardless of the substrate concentrations used (FIG. 2A). Further experiments

using the SssI DNMT showed that when a fixed concentration of AdoMet was present, increasing the concentrations of AdoHcy decreased the  $V_{MAX}$  values for SssI DNMT-mediated DNA methylation in a concentration-dependent manner (FIG. 2B), as determined by using the curve regression method or the Eadie-Hofstee plot. These data suggested that the mechanism of inhibition by AdoHcy was essentially noncompetitive with respect to the formation of the methylated DNA products. This conclusion is consistent with the data in FIG. 2A showing that the inhibition potency and efficacy of AdoHcy were not affected by varying the concentrations of the DNA substrate.

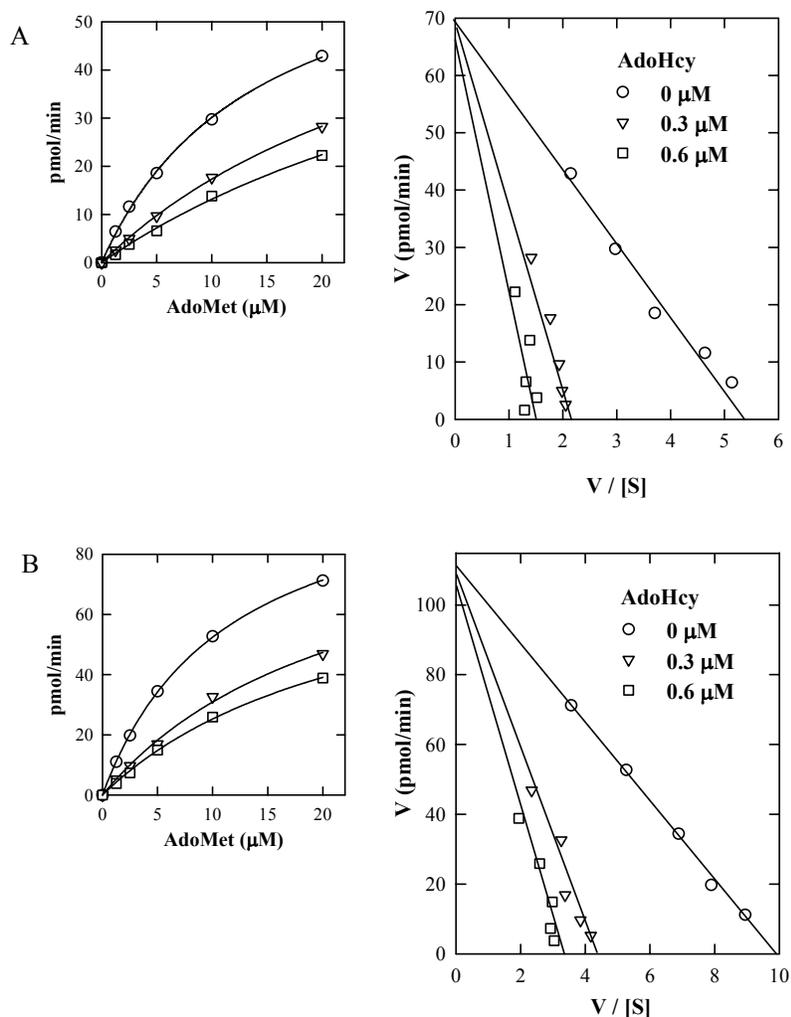


FIGURE 3. EADIE-HOFSTEE PLOT FOR THE INHIBITION OF ADO MET FUNCTION BY ADO HCY. Either in the absence (A) or presence of COMT (B). The left insets showed the rate of DNA methylation as a function of AdoMet concentration. The incubation mixture consisted of 1.0 μM poly(dG-dC)·poly(dG-dC), 0-20 μM AdoMet, 0.5 μCi [methyl-<sup>3</sup>H]AdoMet, 0-0.6 μM AdoHcy, 1 unit of SssI DNMT (lot No. 16-3), and 0 or 2 units of COMT in a final volume of 25 μl. The incubations were carried out at 37°C for 30 min in the presence of 10 mM MgCl<sub>2</sub>. Each data point was derived from at least two replicate measurements.

Further kinetic analysis showed that the presence of AdoHcy (at 0.3 and 0.6 μM) increased the apparent  $K_M$  values of AdoMet for the SssI DNMT-mediated DNA methylation in a concentration-dependent manner, but the original  $V_{MAX}$  value could still be obtained by increasing the concentrations of AdoMet (FIG. 3A). This data suggested that AdoHcy competed with AdoMet for binding with the SssI DNMT, thereby decreasing the apparent binding affinity of AdoMet for the enzyme. We

have conducted similar kinetic studies on mechanism of inhibition of SssI DNMT by AdoHcy in the presence of COMT (at a final concentration of 2 units or 1.44 μg per 25 μL). The overall kinetic pattern for the inhibition by AdoHcy remained almost the same although the magnitude of decrease of  $V_{MAX}$  value in the presence of AdoHcy was slightly less compared to that in the absence of COMT (compare FIG. 3B with FIG. 3A), which was as expected.

Taken together all the kinetic data we have obtained, it is clear that the mechanism by which COMT enhances the SssI DNMT-mediated DNA methylation was through reducing the concentrations of the available unbound AdoHcy, a very potent, high-efficacy inhibitor of the enzymatic methylation of DNA.

3.2. MODULATION OF HUMAN DNMT1-MEDIATED DNA METHYLATION BY COMT

For comparison, we have also determined the modulating effects of COMT on human DNMT1-

mediated methylation of the poly(dI-dC)·poly(dI-dC) substrate by altering the amounts of COMT (0.5–8 units, i.e., 0.36–5.73  $\mu\text{g}$ ) present in the incubation mixture (final volume 25  $\mu\text{L}$ ). The presence of the COMT from 0.5 to 8 units increased, in a concentration-dependent manner, the rate of DNMT1-mediated DNA methylation in vitro (FIG. 4A). The maximal enhancement of the DNMT1-mediated DNA methylation was ~90% over the control values when 8 units (5.73  $\mu\text{g}$ ) of the COMT were present.

Similar to SssI DNMT-mediated DNA methylation described above, the in vitro DNA methylation

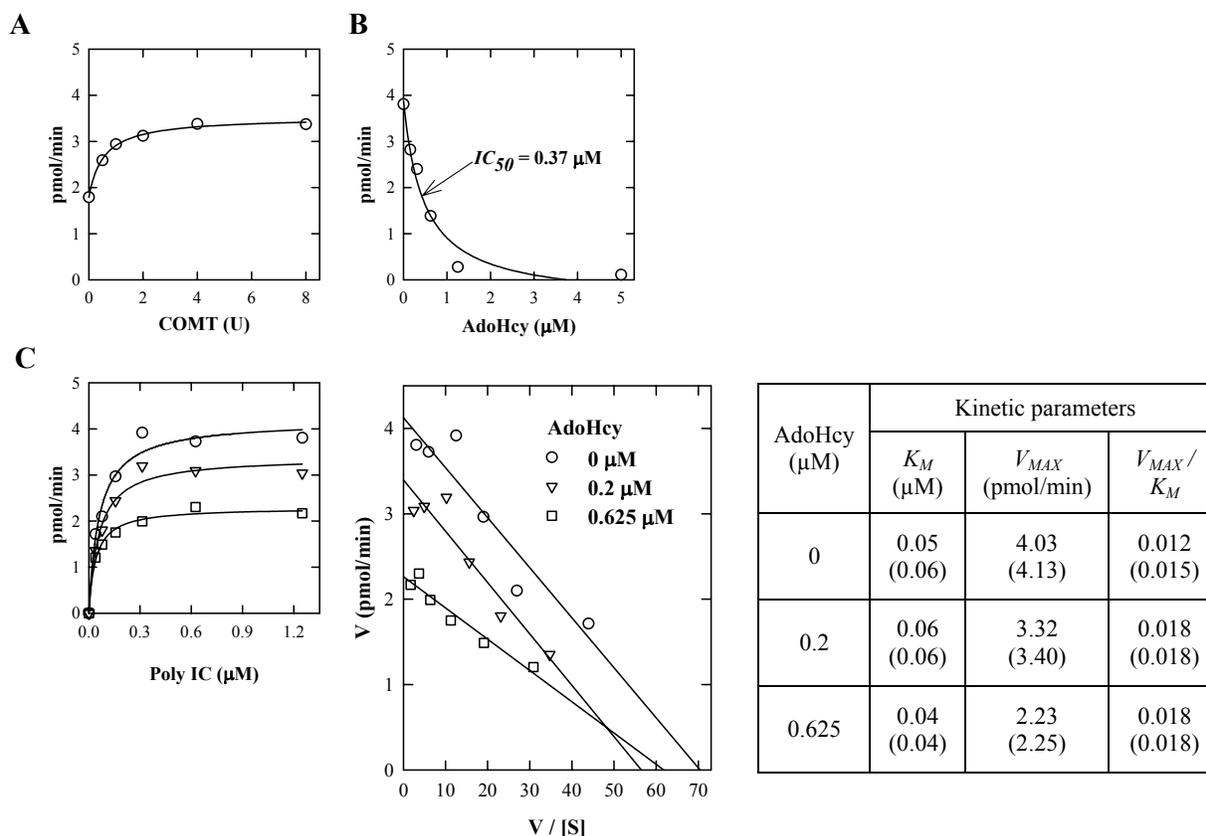


FIGURE 4. THE EFFECTS OF COMT ON THE KINETICS OF HUMAN DNMT1-MEDIATED DNA METHYLATION IN VITRO. A: Concentration dependence for the enhancement of DNA methylation by COMT. The incubation mixture consisted of 1  $\mu\text{M}$  poly(dI-dC)·poly(dI-dC), 5  $\mu\text{M}$  AdoMet (containing ~0.5  $\mu\text{Ci}$  [methyl- $^3\text{H}$ ]AdoMet), 1 unit of DNMT1, and 0.5-8 units of COMT in a final volume of 25  $\mu\text{L}$ . B: Inhibition of DNMT1-mediated DNA methylation in the absence of COMT. AdoHcy concentrations used were 0.15, 0.315, 0.625, 1.25, and 5.0  $\mu\text{M}$ . C: The left panel illustrates the rate of DNMT1-mediated DNA methylation in the absence or presence of AdoHcy, and the right panel shows the Eadie-Hofstee plot of the same data set. D: The  $K_M$  and  $V_{MAX}$  values calculated according the data shown in panel C. Note that the kinetic parameters were calculated by using both the curve-fitting functions of the SigmaPlot software and the Eadie Hofstee plots (the numbers in parenthesis). The concentrations of poly(dI-dC)·poly(dI-dC) were 0.039, 0.078, 0.156, 0.313, 0.625, and 1.25  $\mu\text{M}$ . The batch number of the human DNMT1 used to generate the data in A and B was 15-6, and that for the data in C was 17-12. Incubations were carried out at 37°C for 30 min in the absence of  $\text{MgCl}_2$ . Each data point was derived from at least two replicate measurements.

by human DNMT1 was inhibited by AdoHcy in a concentration-dependent manner, and the estimated  $IC_{50}$  values for AdoHcy were  $<0.5 \mu\text{M}$  (FIG. 4B). Altering the concentrations of the poly(dI-dC)·poly(dI-dC) substrate did not alter the inhibition potency and efficacy of AdoHcy, suggesting that AdoHcy was a noncompetitive inhibitor. The rates of methylation of poly(dI-dC)·poly(dI-dC) (at 0.04–1.25  $\mu\text{M}$  concentrations) in the absence of AdoHcy followed of a typical hyperbolic curve pattern and reached plateau rates at 0.3–0.6  $\mu\text{M}$  DNA substrate concentrations (FIG. 4C), with  $K_M$  of 0.06  $\mu\text{M}$  and  $V_{MAX}$  of 4.13 pmol/min. When a fixed concentration of AdoMet was present, increasing the concentra-

tions of AdoHcy decreased the  $V_{MAX}$  values for the DNMT1-mediated DNA methylation in a concentration-dependent manner but the  $K_M$  values were essentially not altered (FIG. 4C, 4D). The curve regression analysis and Eadie-Hofstee plots both showed that the mechanism of inhibition by AdoHcy was essentially noncompetitive with respect to the formation of methylated DNA products.

### 3.3. EFFECT OF COMT siRNAs ON THE DNA METHYLATION STATUS IN CULTURED MCF-7 CELLS

When two different concentrations (50 and 100 nM) of the specifically-designed siRNAs for the

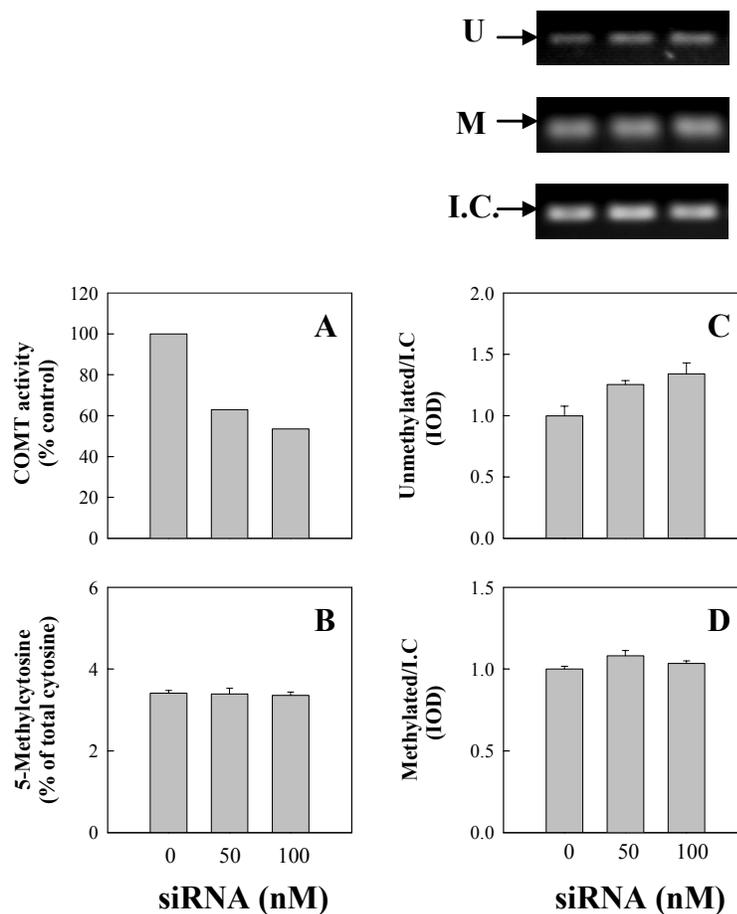


FIGURE 5. THE EFFECTS OF TRANSFECTIONS WITH THE COMT siRNAs ON COMT EXPRESSION AND ON DNA METHYLATION IN MCF-7 CELLS. A: Inhibition of COMT activity after three transfections with the COMT siRNAs in a period of 10 days. Each value was the mean from two representative samples. B: The status of the global DNA methylation status following transfections with the COMT siRNAs. Each value was the mean  $\pm$  S.E. of 3-4 measurements (N = 4 for the control, and N = 3 for the siRNAs-treated groups). C and D: Alterations of the unmethylation-specific band and methylation-specific band, respectively, of the RAR $\beta$  gene after transfections with the COMT siRNAs. Each value was the mean  $\pm$  S.E. of 3-4 determinations. One representative set of the unmethylation-specific band (U), the methylation-specific band (M), and the internal control (IC) is also shown for reference.

human COMT gene were transfected into cultured MCF-7 cells, the rate of O-methylation of quercetin by cell lysates was decreased in a concentration-dependent manner (FIG. 5A). The maximal decrease in the overall COMT enzymatic activity in MCF-7 cells 10 days following three consecutive transfections with 100 nM COMT siRNAs was ~50%, suggesting that the COMT gene expression was partially suppressed by the COMT siRNAs. By using the isolated DNA from these cells, we also determined the effect of COMT siRNA treatments on the global DNA methylation status in these cells. Our data showed that the COMT siRNAs did not have an appreciable effect on the global content of 5-methylcytosine in the DNA. We then also determined the effect of COMT siRNAs on the methylation status of the RAR $\beta$  gene in MCF-7 cells. It is known that the RAR $\beta$  gene was extensively hypermethylated in these cells [21]. We observed that following 3 consecutive transfections of these cells with 50 or 100 nM of the COMT siRNAs in a period of 10 days, the unmethylation-specific band for the RAR $\beta$  gene was slightly increased (up to 25%) in a concentration-dependent manner. In comparison, corresponding changes in the methylation-specific band for the RAR $\beta$  gene were not detected. Here it should also be noted that the relative intensity of the methylation- and unmethylation-specific bands for the RAR $\beta$  gene as shown in FIG. 5C did not exactly reflect the real ratio between the methylated and unmethylated copies of the RAR $\beta$  gene, because this ratio might have been unproportionally altered during the 2-stage nested PCR amplifications of the targeted sequences.

#### 4. DISCUSSION

The results of our present study demonstrated, for the first time, that the presence of COMT at physiologically-relevant concentrations enhanced the rate of DNA methylation in vitro catalyzed by the prokaryotic SssI DNMT as well as the human DNMT1, with an enhancement of up to 1-fold over the control rates. Enzyme kinetic analyses showed that AdoHcy strongly and noncompetitively inhibited the methylation of DNA by competing AdoMet off the DNMTs and thereby shifting more

DNMT molecules to an inactive state which were bound with AdoHcy. Consequently, the  $V_{MAX}$  values were decreased by AdoHcy in a concentration-dependent manner, but the  $K_M$  values were basically not changed. Similarly, when COMT was present, the  $V_{MAX}$  values were increased but the  $K_M$  values still remained basically unchanged. Taken together, our kinetic analyses suggest that the enhancement of DNA methylation by COMT likely is largely due to the sequestration of AdoHcy by COMT, which reduces the availability of the free AdoHcy for inhibition of the DNMTs.

It is of note that while the  $K_M$  values of AdoMet for SssI DNMT- and human DNMT1-mediated DNA methylation were found to be 3.5 and 9.2  $\mu$ M, respectively (data not presented), the  $IC_{50}$  values of AdoHcy for these two DNMTs were much lower, 0.31 and 0.37  $\mu$ M, respectively (FIG. 1 AND FIG. 4). These data clearly suggested that each of these two DNMTs had a 10 to 30-fold higher apparent binding affinity for AdoHcy than for AdoMet. Since the  $IC_{50}$  values for the inhibition of DNMT1-mediated DNA methylation by AdoHcy are lower than the available cellular or circulating concentrations of AdoHcy in humans [22], this data would suggest that AdoHcy likely is one of the major inhibitory regulators of the rate of cellular DNA methylation in vivo.

In order to probe whether alterations of the cellular COMT levels could modulate the DNA methylation status, we also studied the effects of the specifically-designed siRNAs for the human COMT gene on the global DNA methylation status as well as on the gene-specific methylation in cultured MCF-7 cells. When the COMT activity in MCF-7 cells was reduced ~50% by transfections with siRNAs, the global content of 5-methylcytosine in the DNA was not significantly altered. However, the decreases of the cellular COMT activity were correlated with small increases ( $P < 0.05$ ) in the unmethylation-specific band for the RAR $\beta$  gene. In comparison, significant changes in the methylation-specific band for the RAR $\beta$  gene were not detected. The lack of detectable changes in the methylation-specific band likely can be explained by the fact that the RAR $\beta$  gene in MCF-7 cells is mostly methylated [21]. As such, a small decrease in the methylation status of this gene may not be readily reflected in the optical density of the methylation-specific band. In line with this suggestion, our data indeed showed

that the increase in the optical density of the unmethylation-specific band was only ~25% above the controls, and this would mean that there was only a rather small decrease in the overall methylation status of the RAR $\beta$  gene after the cells had been cultured for 10 days under a condition where the expression of the COMT gene was reduced by ~50%.

Although COMT is known to be almost ubiquitously present in the body, some tissues or cells (such as liver, kidney, and intestinal epithelial cells) expressed much higher levels of this enzyme than other tissues. While the presence of higher levels of COMT may increase the rate of cellular DNA methylation, the elevated levels of this enzyme may also lead to an increase of the enzymatic O-methylation of various catechol substrates, which would lead to increased formation of AdoHcy. Under certain conditions such as when elevated amounts of dietary catechol substrates are ingested into the body, increased methylation metabolism of these catechols by COMT is expected to cause an inhibition of the DNA methylation due to increased formation of AdoHcy. Therefore, it may be difficult to directly predict the rate of DNA methylation solely based on the cellular COMT levels alone, and other important factors (such as the amount of the COMT substrates present in the body) should also be considered.

In summary, the results of our present study demonstrated that the presence of COMT at physiologically-relevant concentrations enhanced the rate of DNA methylation in vitro catalyzed by SssI DNMT and human DNMT1, with an enhancement of up to 1-fold over the control rate. In cultured MCF-7 cells, decreased expression of COMT for a short period of 10 days resulted in small decreases in the overall methylation status of the RAR $\beta$  gene. Because AdoHcy is an extremely potent noncompetitive inhibitor of DNMTs, the enhancement of enzymatic DNA methylation by COMT is believed to be due to the sequestration of AdoHcy by COMT, which reduces the availability of free AdoHcy for causing DNMT inhibition.

## ACKNOWLEDGEMENT

The authors are indebted to Dr. Won Jae Lee at the Ewha Womans University (Seoul, South Korea) for his expert assistance with the techniques used in the present study.

## REFERENCES

- [1] CHEN RZ, PETTERSSON U, BEARD C, JACKSON-GRUSBY L AND JAENISCH R [1998] DNA hypomethylation leads to elevated mutation rates. *Nature* 395: 89-93.
- [2] LI E, BEARD C AND JAENISCH R [1993] Role for DNA methylation in genomic imprinting. *Nature* 366: 362-365.
- [3] LI E, BESTOR TH AND JAENISCH R [1992] Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69: 915-926.
- [4] OKANO M, BELL DW, HARBER DA AND LI E [1999] DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99: 247-257.
- [5] BEARD C, LI E AND JAENISCH R [1995] Loss of methylation activates Xist in somatic but not embryonic cells. *Genes Dev* 9: 2325-2334.
- [6] ESTELLER M [2002] CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 21: 5427-5440.
- [7] LIN CH, HSIEH SY, SHEEN IS, LEE WC, CHEN TC, SHYU WC AND LIAW YF [2001] Genome-wide hypomethylation in hepatocellular carcinogenesis. *Cancer Res* 61: 4238-4243.
- [8] Esteller M and Herman JG [2002] Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumors. *J Pathol* 196: 1-7.
- [9] HERMAN JG AND BAYLIN SB [2003] Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 349: 2042-2054.
- [10] STIRZAKER C [1997] Extensive DNA methylation spanning the Rb promoter in retinoblastoma tumors. *Cancer Res* 57: 2229-2237.
- [11] OKANO M, XIE S AND LI E [1998] Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 19: 219-220.
- [12] UELAND PM [1982] Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase. *Pharmacol Rev* 34: 223-253.
- [13] ZHU BT AND LIEHR JG [1996] Inhibition of catechol O-methyltransferase-catalyzed O-methylation of 2- and 4-hydroxyestradiol by quercetin: Possible role in estradiol-induced tumorigenesis. *J Biol Chem* 271: 1357-1363.
- [14] ZHU BT, PATEL UK, CAI MX AND CONNEY AH [2000] O-Methylation of tea polyphenols catalyzed by human

- placental cytosolic catechol-O-methyltransferase. *Drug Metab Dispos* 28: 1024-1030.
- [15] ZHU BT, PATEL UK, CAI MX, LEE AJ AND CONNEY AH [2001] Rapid conversion of tea catechins to monomethylated products by rat liver cytosolic catechol-O-methyltransferase. *Xenobiotica* 31: 879-890.
- [16] RENBAUM P, ABRAHAMOVE D, RAINSDO A, WILSON GG, ROTTEM S AND RAZIN A [1990] Cloning, characterization, and expression in *Escherichia coli* of the gene coding for the CpG DNA methylase from *Spiroplasma* sp. strain MQ1(M.SssI). *Nucleic Acids Res* 18: 1145-1152.
- [17] LIU ZJ AND ZHU BT [2004] Concentration-dependent mitogenic and antiproliferative actions of 2-methoxyestradiol in estrogen receptor-positive human breast cancer cells. *J Steroid Biochem Mol Biol* 88: 265-275.
- [18] ZHU BT, EZELL EL AND LIEHR JG [1994] Catechol-O-methyltransferase-catalyzed O-methylation of mutagenic flavonoids: Metabolic inactivation as possible reason for their lack of carcinogenicity in vivo. *J Biol Chem* 269: 292-299.
- [19] RAMSAHOYE BH [2002] Measurement of genome wide DNA methylation by reversed-phase high performance liquid chromatography. *Methods* 27: 156-161.
- [20] PALMISANO WA, DIVINE KK, SACCOMANNO G, GILLILAND FD, BAYLIN SB, HERMAN JG AND BELINSKY SA [2000] Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res* 60: 5954-5958.
- [21] SIRCHIA SM, FERGUSON AT, SIRONI E, SUBRAMANYAN S, ORLANDI R, SUKUMAR S AND SACCHI N [2000] Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor  $\beta_2$  promoter in breast cancer cells. *Oncogene* 19: 1556-1563.
- [22] MELNYK S, POGRIBNA M, POGRIBNY IP, YI P AND JAMES SJ [2000] Measurement of plasma and intracellular S-adenosylmethionine and S-adenosylhomocysteine utilizing coulometric electrochemical detection: Alterations with plasma homocysteine and pyridoxal 5'-phosphate concentration. *Clin Chem* 46: 265-272.

RECEIVED ON 10-5-2004.

ACCEPTED ON 12-12-2004.