Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols

Won Jun Lee and Bao Ting Zhu*

Department of Basic Pharmaceutical Sciences, College of Pharmacy, University of South Carolina, Columbia, SC 29208, USA

*To whom requests for reprints and correspondence should be addressed at: 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. Tel: 803 777 4802; Fax: 803 777 8356; Email: BTZhu@cop.sc.edu

We studied the modulating effects of caffeic acid and chlorogenic acid on the in vitro methylation of synthetic DNA substrates and also on the methylation status of the promoter region of a representative gene in two human cancer cell lines. Under conditions that were suitable for the in vitro enzymatic methylation of DNA and dietary catechols, we found that the presence of caffeic acid or chlorogenic acid inhibited in a concentration-dependent manner the DNA methylation catalyzed by prokaryotic M.SsSI DNA methyl transferase (DNMT) and human DNMT1. The IC50 values of caffeic acid and chlorogenic acid were 3.0 and 0.75 μM, respectively, for the inhibition of M.SsSI DNMT-mediated DNA methylation, and were 2.3 and 0.9 μM, respectively, for the inhibition of human DNMT1-mediated DNA methylation. The maximal in vitro inhibition of DNA methylation was ~80% when the highest concentration (20 μM) of caffeic acid or chlorogenic acid was tested. Kinetic analyses showed that DNA methylation catalyzed by M.SsSI DNMT or human DNMT1 followed the Michaelis–Menten curve patterns. The presence of caffeic acid or chlorogenic acid inhibited DNA methylation predominantly through a non-competitive mechanism, and this inhibition was largely due to the increased formation of S-adenosyl-L-homocysteine (SAH, a potent inhibitor of DNA methylation), resulting from the catechol-O-methyltransferase (COMT)-mediated O-methylation of these dietary catechols. Using cultured MCF-7 and MAD-MB-231 human breast cancer cells, we also demonstrated that treatment of these cells with caffeic acid or chlorogenic acid partially inhibited the methylation of the promoter region of the RARβ gene. The findings of our present study provide a general mechanistic basis for the notion that a variety of dietary catechols can function as inhibitors of DNA methylation through increased formation of SAH during the COMT-mediated O-methylation of these dietary chemicals.

Introduction

DNA methylation at the 5-position of cytosine within the CpG dinucleotides represents an important mechanism for the epigenetic control of gene expression and the maintenance of genome integrity. Global hypomethylation, accompanied by region-specific hypermethylation, is a common characteristic in tumor cells. While global genomic hypomethylation may be associated with the induction of chromosomal instability (1), gene-specific hypermethylation is known to be associated with the inactivation of various pathways involved in the tumorigenic process, including DNA repair (2), cell cycle regulation (3,4), inflammatory/stress response (5) and apoptosis (6). A number of recent studies have suggested that bioactive food components, including both essential and non-essential nutrients, can modify DNA methylation patterns in complex ways (7). A recent study by Fang et al. (8) showed that tea catechins are effective inhibitors of human DNMT-mediated DNA methylation in vitro and also in cultured cancer cells.

DNA methylation is catalyzed by specific DNA methyltransferases (DNMTs), using S-adenosyl-L-methionine (SAM) as the methyl donor. Multiple DNMTs appear to be present in humans, with varying degrees of specificity toward unmethylated and hemi-methylated DNA substrates. Presently, four mammalian DNMTs, including the DNMT1 (9), DNMT2 (10), DNMT3A and DNMT3B (11), have been identified. DNMT1 has a 7- to 21-fold preference for hemi-methylated DNA substrates than unmethylated DNA substrates, and it is largely responsible for copying the methylation pattern of the parental strand to the daughter strand during the DNA replication process (12). In comparison, DNMT3A and 3B enzymes have similar affinities for both unmethylated and hemi-methylated DNA substrates (11), and they are expressed at high levels during the early stages of embryonic development and are primarily responsible for the formation of de novo DNA methylation patterns (11).

It is known that various catechol-containing dietary polyphenols are excellent substrates for the COMT-mediated methylation (13–16). The COMT-mediated rapid methylation of large amounts of dietary catechols would not only significantly drain the intracellular pools of SAM, but it would also form equimolar amounts of S-adenosyl-L-homocysteine (SAH), which is the demethylated SAM and is also a feedback inhibitor of various SAM-dependent methylation processes. Given that the DNMT-mediated methylation of DNA and the COMT-mediated O-methylation of catechols use the same pools of the methyl donor SAM for their reactions and both contribute to the formation of SAH as a feedback inhibitor, it is reasonable to suggest that the amount of various catecholic dietary polyphenols ingested may be an important cumulative modulator of the cellular DNA methylation process (depicted in Figure 1), and this possibility was investigated in the present study by determining the effects of two catechol-containing coffee polyphenols (caffeic acid and chlorogenic acid).

Abbreviations: COMT, catechol-O-methyltransferase; DAC, 5-aza-2’-deoxycytidine; DNMT, DNA methyltransferase; IOD, integrated optical density; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine.
cocktail consisting of 4-(2-aminoethyl)benzenesulfonyl fluoride, peptatin A, E64, bestatin, leupeptin and aprotonin. It should be noted that different batches of the enzymes gave quite different overall catalytic activities and $V_{\text{MAX}}$ values (up to 5-fold), but the $K_m$ values of the enzymes appeared to be quite consistent. [Methyl-$^3$H]SAM (sp. act. of 11.2–13.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The ScintiVerse BD scintillation cocktail was obtained from Fisher Scientific (Pittsburgh, PA).

**Assay of enzymatic DNA methylation in vitro**

The following reaction mixtures were freshly prepared on ice immediately before 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 μM of the CpG methylation sites. Note that 1 mol of the double-stranded dG–dC·dG–dC and dI–dC·dI–dC dinucleotides was considered to contain 2 mol of the CpG methylation sites. The stock solutions of M.SssI DNMT and DNMT1 were further diluted in water, usually to a concentration of 1 U/5 μl. The methyl donor SAM (containing ~0.5 μCi [methyl-$^3$H]SAM) was dissolved in 0.8 mM HEPES at desired stock concentrations depending on the experiments. As recommended by the supplier of the DNMTs, the reaction buffer for M.SssI DNMT comprised of 50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl₂, 1 mM dihydrothioretol (pH 7.4), and the reaction buffer for DNMT1 comprised of 50 mM Tris–HCl, 1 mM EDTA, 1 mM dihydrothioretol and 5% glycerol (pH 7.8).

The above freshly-prepared solutions were then added in a sequential order into a 1.5 ml microcentrifuge tube on ice: 5 μl each of the reaction buffer, synthetic DNA substrate, enzyme, water and SAM (mixed with [methyl-$^3$H]-SAM). Notably, in some of the experiments designed to study the effects of COMT and dietary chemicals, the 5 μl volume of water was replaced with 2.5 μl of COMT and 2.5 μl of a dietary chemical (added separately to the reaction mixture). As such, the final reaction mixture usually contained 0.125–10 μM of the CpG methylation sites, 1.25–20 μM of SAM (containing ~0.5 μCi [methyl-$^3$H]-SAM) and 0.25–4 U of the enzyme in a final volume of 25 μl. Since Mg²⁺ ion is a necessary cofactor for the activity of COMT, MgCl₂ (at a final concentration of 2 mM) was added into the human DNMT1-mediated reaction tube, as the corresponding reaction buffer supplied by the company did not contain MgCl₂. The incubations were carried out at 37°C for varying lengths of time, and the reactions were arrested by immediately placing the tubes on ice, followed by the addition of 145 μl ice-cold 0.9% sodium chloride and 100 μl salmon testes DNA (at 1 mg/ml in the TE buffer). To precipitate the DNA, 30 μl of 3 M sodium acetate and 900 μl ice-cold ethanol (95%) were added, and the samples were placed in a –80°C freezer for 2 h. After centrifugation at ~10 000 g for 10 min, 100 μl of the heat-deactivated flour (suspended at 40 mg/ml in double-distilled water) was added to each tube, followed by centrifugation at ~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 tube, which would prevent the pellet from being partially washed away during the following washing steps. Notably, comparison of various measurements showed that the addition of flour made the intra-assay as well as inter-assay variations much smaller when compared with the parallel assays without flour. The pellets were then gently washed three 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 μl of 100% ethanol and 1 ml of TE buffer (pH 7.6). Each vial was sonicated for ~10 min and vortexed thoroughly to assure adequate resuspension of the pellet, and then the content was transferred into a scintillation vial (containing 4 ml of ScintiVerse BD) for the measurement of $^3$H-radioactivity, using 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 U) of the M.SssI DNMT and DNMT1 was used in most of the assays (unless otherwise indicated), the rate of DNA methylation, mediated by M.SssI DNMT or human DNMT1, was thus expressed as ‘pmol of methylated DNA’ per min (‘pmol/min’). The kinetic parameters ($K_m$ and $V_{\text{MAX}}$) were calculated manually, according to the Eadie–Hofstee plots (I versus $V$) [5].

**Determination of the methylation status of the RARβ gene in cultured cancer cells**

Three human breast cancer cell lines (MCF-7, MDA-MB-231 and T-47D) were used in this study and they were obtained from the American Type Culture Collection (Manassas, VA). The culture of these cells was described in detail in our recent study (19). The MCF-7 cells were treated with 1, 5, 20 or 50 μM of caffeic acid or chlorogenic acid or 5 μM of 5-aza-2′-deoxycytidine (DAC) for 8 days, and the MDA-MB-231 cells were treated with 0.2, 1, 5 or 20 μM of caffeic acid or chlorogenic acid or 5 μM of DAC for 3 days.
T-47D cells were treated with 20 or 50 μM of caffeic acid or chlorogenic acid for 2 days. The cells were then harvested for DNA extraction by using the DNeasy tissue kit (Qiagen, Valencia, CA). The global methylation status was determined by using the reverse-phase HPLC method as described previously (20). For the measurement of gene-specific DNA methylation, the extracted DNA (1 μg) was modified using the EZ DNA methylation kit (Zymo Research, Orange, CA) under conditions specified by the supplier. All unmethylated cytosines were converted to uracils, whereas 5-methylcytosines remained unaltered. The methylation status of the promoter regions of the genes was determined by using a nested two-stage PCR method as described earlier (21). Sodium bisulfite-modified DNAs were first subjected to the Stage-I PCR to amplify the 425 and 208 bp fragments of the RARβ and p16 genes, respectively (Table I). The products include a portion of the CpG-rich promoter region of the gene. The primers (obtained from Invitrogen Life Technologies, Frederick, MD) for the 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 (RARβ) or 1000 (p16) times, and 2.5 μl of the diluted 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 RARβ and p16 genes are summarized in Table I. HotStarTaq DNA polymerase (Qiagen) in a 25-μl reaction volume was used in all the PCRs. The conditions for the Stage-I PCR were as follows: 95°C for 15 min, denaturing at 95°C for 45 s (RARβ) or 30 s (p16), annealing at 53°C for 1 min (RARβ) or 30 s (p16) for 40 cycles followed by a final extension at 72°C for 10 min. Conditions for the Stage-II PCR were 95°C for 15 min, denaturing at 95°C for 30 s (RARβ) or 15 s (p16), annealing at 62°C for 30 s (RARβ) or 15 s (p16), extension at 72°C for 30 s (RARβ) or 15 s (p16) for 18–26 cycles and a final 10-min extension step at 72°C.

The number of cycles and the PCR conditions for the RARβ and p16 genes were individually 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, directly visualized using 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 that served as internal standards for the Stage-I PCR products (depicted in Table I). 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).

### Results

#### Optimization of the conditions for enzymatic DNA methylation in vitro

Prior to testing the effects of various dietary chemicals on DNA methylation, we first optimized the reaction conditions for the in vitro DNA methylation by determining the effects of different incubation time, and the effects of different concentrations of the enzyme (M.SssI DNMT and human DNMT1), of the methyl donor SAM and of the synthetic DNA substrates (Figure 2). Based on our measurements, a common reaction condition was found to be suitable for both M.SssI DNMT- and DNMT1-mediated DNA methylation, which included an incubation time of 30 min, an enzyme concentration of 1 U/25 μl, a SAM concentration of 5 μM and the substrate concentrations from 0.1 to 5 μM. Notably, in order to study the modulating effects of catechol-containing dietary chemicals on the rate of DNA methylation in vitro, COMT was also added to the reaction mixture, in addition to the dietary chemicals (at various concentrations). In the present study, a fixed concentration of porcine liver COMT (usually at 2 U/25 μl for M.SssI DNMT and 4 U/25 μl for human DNMT1) was used because our measurements showed that COMT at these concentrations provided a moderate rate of methylation of the representative catechol substrates, such as catechol estrogens and tea catechins (data not shown).

### Table I. List of primer sequences, annealing temperatures and expected product sizes for MSP

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers (5’-3’)</th>
<th>Reverse primers (5’-3’)</th>
<th>Annealing temp. (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RARβ</td>
<td>N: TTAGGTGTCAGACGATTTTG</td>
<td>CACTAAATTAATCCGACATC ACC</td>
<td>53</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td>IC: AGATAGTGGGTTTATTTGAA</td>
<td>CATCCGATCTCCAACAA</td>
<td>53</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>U: TTGGAAAATGGAGCAGATTTGA</td>
<td>AAACCATCACCACAAAAA</td>
<td>62</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>M: TCGAGAACGGGACGCATTGCG</td>
<td>GACCGATCCACCGGAAA</td>
<td>62</td>
<td>146</td>
</tr>
<tr>
<td>p16</td>
<td>N: GGAAGGGGGGAGATGAGTG</td>
<td>CTCAAACCCTCTACACCT</td>
<td>60</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td>IC: GGGGGATGATGATGATCTTT</td>
<td>CTCAAAACCCTTACACCA</td>
<td>54</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>U: TGGGGGATGATGGGATGTTG</td>
<td>CAACCCCAACCAACAAAA</td>
<td>62</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>M: CGGGGGATGATGAGGATCAGGCG</td>
<td>GACCGGCAACCAGGACCTAA</td>
<td>62</td>
<td>81</td>
</tr>
</tbody>
</table>

N, nest primers; IC, internal control primers; U, unmethylation-specific primers; and M, methylation-specific primers.
Modulation of DNMT-mediated DNA methylation by caffeic acid and chlorogenic acid

When COMT was not present in the reaction mixture, caffeic acid or chlorogenic acid (at 5 or 20 μM) had a relatively weak inhibitory effect on M.SsSI DNMT-mediated DNA methylation (Figure 3A, upper panels). When COMT was present, caffeic acid or chlorogenic acid at the same concentration range exerted a markedly stronger inhibition of DNA methylation, and this inhibition was concentration-dependent (Figure 3A, lower panels). We noted that the maximum inhibition at the highest inhibitor concentration tested (20 μM) was also dependent on the concentrations of SAM present. At 5 μM SAM, caffeic acid or chlorogenic acid had a higher maximum inhibition of DNA methylation than the inhibition observed at 20 μM SAM. The estimated IC50 values of caffeic acid and chlorogenic acid for the inhibition of DNA methylation were ~3.0 and 0.75 μM, respectively, at 5 μM SAM, and were ~13.5 and 2.8 μM, respectively, at 20 μM SAM. These data also indicated that chlorogenic acid had a higher potency and efficacy in inhibiting the M.SssI DNMT-mediated DNA methylation than did caffeic acid.

For comparison, we also determined the inhibitory effect of these two coffee polyphenols on human DNMT1-mediated DNA methylation. Caffeic acid and chlorogenic acid (at 5 or 20 μM) exerted a very weak direct inhibition of human DNMT1-mediated DNA methylation in the absence of COMT (Figure 3B, upper panels). However, in the presence of COMT, DNMT1-mediated DNA methylation was strongly inhibited by caffeic acid and chlorogenic acid in a concentration-dependent manner. At 5 μM SAM, the estimated IC50 values were ~2.3 and 0.9 μM, respectively, suggesting that chlorogenic acid had a 3-fold higher potency than caffeic acid (Figure 3B, lower panels). Moreover, the maximum inhibition by chlorogenic acid at its highest concentration tested (20 μM) was also somewhat greater than that of caffeic acid.

Kinetic mechanisms for the inhibition of DNA methylation by caffeic acid and chlorogenic acid

We determined the apparent kinetic features for the inhibition of enzymatic DNA methylation. When M.SsSI DNMT was assayed as the enzyme, our data showed that the presence of caffeic acid or chlorogenic acid at two very different concentrations (0.625 and 10 μM) caused a clear concentration-dependent decrease in the Vmax values (Figure 4, upper panels), whereas the Kcat values were not markedly or consistently altered, as calculated according to the curve regression analysis or the Eadie–Hofstee plots. Very similar kinetic changes were also observed when the human DNMT1 was used as the enzyme (Figure 4, lower panels). These data clearly suggested a predominantly non-competitive mechanism of inhibition of both M.SsSI DNMT and human DNMT1 by caffeic acid and chlorogenic acid, particularly at higher inhibitor concentrations.

To gain a better understanding of the kinetic mechanism(s) for the observed inhibition of DNA methylation by dietary catechols, we also determined the effects of SAH on M.SsSI

**Fig. 2.** Optimization of the conditions for the in vitro DNA methylation catalyzed by the M.SsSI DNMT (upper panel) and human DNMT1 (lower panel). Upper panel: (A) Poly CG (at 5 μM) was incubated with M.SsSI DNMT (at 0.25, 0.5, 1, 2 and 4 U/25 μl) and 20 μM [methyl-3H]SAM. (B) M.SsSI DNMT (1 U/25 μl) was incubated with poly CG (0.1, 0.5, 0.625, 2.5, 5 and 10 μM) and 20 μM [methyl-3H]SAM. (C) A concentration range of 1.25–20 μM [methyl-3H]SAM was used in reactions containing 1 μM poly CG and 1 U/25 μl of M.SsSI DNMT. (D) The time course for the methylation of 1 μM poly CG by 1 U/25 μl of M.SsSI DNMT in the presence of 10 μM [methyl-3H]SAM. The batch number of M.SsSI DNMT used for generating the data in A, B and C was 16–10, and that for the data in D was 16–3. Lower panel: (A) A concentration range of 0.25–4 U/25 μl DNMT1 was used in reactions containing 5 μM poly IC and 5 μM [methyl-3H]SAM. (B) Different concentrations of poly IC (0.20–10 μM) was incubated with 5 μM [methyl-3H]SAM and 1 U/25 μl of DNMT1. (C) A concentration range of 1.0–40 μM [methyl-3H]SAM was incubated with 1 μM poly IC and 1 U of DNMT1. (D) The time course for the methylation of 1 μM poly IC by 1 U/25 μl of DNMT1 in the presence of 10 μM [methyl-3H]SAM. Each data point is the mean of duplicate measurements.
DNMT-mediated DNA methylation. We found that SAH was a very potent inhibitor of the M.SssI DNMT-mediated DNA methylation \textit{in vitro}, and the estimated IC$_{50}$ values for SAH were $\sim 0.25$ $\mu$M, regardless of the substrate concentrations (Figure 5, upper panel). When a fixed concentration of SAM was present, the increase in the concentrations of SAH decreased the $V_{\text{MAX}}$ values for the M.SssI DNMT-mediated DNA methylation in a concentration-dependent manner (Figure 5, lower panels), indicating that SAH is a pure non-competitive inhibitor with respect to the formation of methylated DNA products. Based on these observations, it is suggested that the mechanism by which the catechol-containing coffee polyphenols inhibited the enzymatic DNA methylation \textit{in vitro} largely resulted from the increased formation of SAH (a potent non-competitive inhibitor of DNMTs) during the COMT-mediated $O$-methylation of these dietary catechols.

**Fig. 3.** Inhibitory effects of caffeic acid and chlorogenic acid on DNA methylation, catalyzed by M.SssI DNMT (A) and human DNMT1 (B). The amount of COMT used during the M.SssI DNMT- and human DNMT1-mediated DNA methylation reaction was 2 and 4 U, respectively, in a final reaction volume of 25 $\mu$l. The final concentrations of MgCl$_2$ for M.SssI DNMT- and DNMT1-mediated DNA methylation were 10 and 2 mM, respectively, and the concentrations of caffeic acid and chlorogenic acid were at 0.625–20 $\mu$M. Each data point was derived from duplicate measurements.

**Fig. 4.** Michaelis–Menten curves for the enzymatic DNA methylation \textit{in vitro} in the absence or presence of caffeic acid or chlorogenic acid. The data in the upper two panels (A and B) were obtained with the prokaryotic M.SssI DNMT, in the presence of 2 U of COMT (in 25 $\mu$l) and 10 mM MgCl$_2$, and the data in the two lower panels (C and D) were obtained with the human DNMT1, in the presence of 4 U of COMT (in 25 $\mu$l) and 2 mM MgCl$_2$. Each data point was derived from at least two duplicate measurements. Different vials of the enzyme preparations obtained from the same supplier were used in the experiments.

Inhibition of the promoter region DNA methylation in cultured cells by caffeic acid and chlorogenic acid

Earlier studies have indicated that the RAR$\beta$ gene promoter region in MCF-7 and MDA-MB-231 cells was hypermethylated (22). Using these cell lines as a model, we examined in this study the effects of caffeic acid and chlorogenic acid on the methylation status of the promoter region of this representative gene. Morphologically, the MCF-7 cells treated with caffeic acid or chlorogenic acid at concentrations up to 50 $\mu$M showed little sign of cytotoxicity (data not shown). Based on the measurement of the amount of total isolated DNA from
each treatment group of the cultured MCF-7 cells, the growth of MCF-7 cells appeared to be not significantly affected by caffeic acid or chlorogenic acid at up to 20 \( \mu M \) concentrations, and was only slightly inhibited (5\%\,-15\%) at the 50 \( \mu M \) concentration (data not shown). Similarly, treatment of MDA-MB-231 cells with caffeic acid or chlorogenic acid at up to 20 \( \mu M \) (the highest concentration tested) also did not appear to markedly affect their morphology and growth (data not shown).

We found that both methylation-specific and unmethylation-specific bands for the \( RAR\beta \) promoter region were detected in cultured MCF-7 and MDA-MB-231 cells prior to treatment with caffeic acid or chlorogenic acid. Treatment of MCF-7 human breast cancer cells with 1, 5, 20 or 50 \( \mu M \) of caffeic acid or chlorogenic acid for 8 days demethylated the hypermethylated \( RAR\beta \) gene in a dose-dependent manner, and increased the unmethylation-specific bands of the \( RAR\beta \) gene (Figure 6). The change of the methylation status of the \( RAR\beta \) gene by treatment with 0.2, 1, 5 or 20 \( \mu M \) of caffeic acid or chlorogenic acid in MDA-MB-231 cells was similar to that observed in MCF-7 cells. The methylation-specific band for the \( RAR\beta \) gene after treatment with caffeic acid or chlorogenic acid for 3 days was decreased, while the unmethylation-specific band of the \( RAR\beta \) gene was increased (Figure 6). We also observed that DAC, a well-known inhibitor of DNA methylation (23), had the strongest effect on both unmethylation-specific and methylation-specific bands in MCF-7 and MDA-MB-231 cells (Figure 6). A similar experiment using cultured T-47D human breast cancer cells also showed that treatment with chlorogenic acid (at 20 and 50 \( \mu M \)) increased the unmethylation-specific band for the promoter region of the \( p16 \) gene, while changes in the methylation-specific band of this gene were less pronounced (data not shown).

In addition, we also used the DNA isolated from MDA-MB-231 cells to probe whether or not the global DNA methylation status in these cells was altered. Quite surprisingly, we did not detect any appreciable changes in the global DNA methylation status following the treatment of MDA-MB-231 cells with either caffeic acid or chlorogenic acid (data not shown).
The results of our present study demonstrated, for the first time, that caffeic acid and chlorogenic acid (two common catechol-containing coffee polyphenols) are strong inhibitors of DNA methylation in vitro, with apparent IC50 values of 3.0 and 0.75 μM, respectively, for inhibition of M.SsSI DNMT-mediated DNA methylation (Figure 3A), and 2.3 and 0.9 μM, respectively, for inhibition of DNMT1-mediated DNA methylation (Figure 3B). Notably, the presence of COMT strongly enhanced the inhibition potency of both caffeic acid and chlorogenic acid compared with the direct inhibition exerted by these two dietary compounds in the absence of COMT. These data suggested that the COMT-mediated methylation of caffeic acid and chlorogenic acid contributed to their higher potency and efficacy of inhibition of DNA methylation in vitro.

Mechanistically, it is suggested that caffeic acid and chlorogenic acid (two representative catechol-containing dietary polyphenols) may inhibit enzymatic DNA methylation through the increased formation of SAH during COMT-mediated O-methylation of dietary catechols. This mechanistic explanation is strongly supported by the following lines of evidence. First, earlier studies by us and also by others have shown that many of the dietary catechol-containing polyphenols are excellent substrates for the COMT-mediated O-methylation (13–16). Metabolic O-methylation of dietary catechols would result in increased formation of SAH. Secondly, the results of our present study showed that SAH is a very potent inhibitor of DNMT-mediated DNA methylation in vitro, with IC50 values <1 μM. Notably, several earlier studies have also shown that SAH is an inhibitor of the DNMT-mediated DNA methylation (24–26). Our enzyme kinetic studies further showed that SAH is a pure non-competitive inhibitor of the DNMT-catalyzed DNA methylation. Thirdly, our enzyme kinetic studies showed that caffeic acid and chlorogenic acid each functioned predominantly as a non-competitive inhibitor of the DNMT-mediated DNA methylation by decreasing the VMAX values in a concentration-dependent manner, yet without markedly affecting the KM values. Lastly, it is worth noting that an earlier study reported that caffeine (which is not a substrate for the COMT-mediated O-methylation) did not show detectable activity for inhibition of DNA methylation in a cell culture system, whereas several other chemicals (modulators of the one carbon cycle) were found to be effective inhibitors (27). This observation is also in line with the
suggested role of the COMT-mediated methylation in mediating the inhibitory effect of some dietary chemicals on DNA methylation.

Earlier studies have indicated that the promoter region of the \textit{RAR\beta} gene in MCF-7 and MDA-MB-231 cells was hypermethylated (22). By using cultured MCF-7 and MDA-MB-231 human breast cancer cells, we showed that treatment of these cells with caffeic acid or chlorogenic acid decreased the methylation status of the promoter region of the \textit{RAR\beta} gene, whereas the unmethylation status of this gene was increased by these treatments (Figure 6). However, we did not see any detectable changes in the global DNA methylation after treatment of one of these two cell lines (MDA-MB-231) with caffeic acid or chlorogenic acid. It is possible that the changes of DNA methylation observed in the present may be mostly gene-specific, and their contribution to the global DNA methylation might be very small and was not detected using the current assay method. Further studies are needed to determine why some genes in a cell are affected quite readily while the current assay method. Further studies are needed to determine gene-specific, and their contribution to the global DNA methylation of one of these two cell lines (MDA-MB-231) with detectable changes in the global DNA methylation after treatment of human breast cancer cells, we showed that treatment of these cells with caffeic acid or chlorogenic acid is consistent with our kinetic data showing a predominant SAH-mediated inhibition of the DNMT1-mediated DNA methylation, it is likely that the increase in the intracellular SAH levels is the predominant contributing factor for the observed inhibition of DNMT methylation. This suggestion is consistent with our kinetic data showing a predominant non-competitive mechanism of inhibition by caffeic acid or chlorogenic acid. Here, it is also worth pointing out that because the IC_{50} values of SAH for the inhibition of DNMT1-mediated DNA methylation appeared to be below the usual cellular or circulating concentrations of SAH, SAH probably is a dominant constitutive inhibitory regulator of DNA methylation \textit{in vivo}.

It has been suggested that hypermethylation of DNA is a key epigenetic mechanism for silencing of various genes, including those encoding the tumor suppressor proteins, DNA repair enzymes and receptors (6, 28–30). A number of recent studies have explored the intriguing possibility of combining a DNMT inhibitor (such as DAC or zebularine) with a histone deacetylase inhibitor (such as trichostatin) as a cancer chemotherapy regimen (31–37). In addition, since several tumor suppressor and receptor genes were reported to be transcriptionally silenced by the hypermethylation during cancer development, it is possible that the inhibition of DNMTs and the histone deacetylase may be a possible mechanism that jointly contributes to the cancer protective actions of certain dietary or chemical agents.

The findings of our present study provide a general mechanism that may contribute to the inhibition of DNA methylation by various dietary catechol-containing polyphenols. While we understand that the usual daily intake of caffeic acid or chlorogenic acid alone may not represent an overwhelming burden for the body’s COMT-mediated methylation system as well as for the intracellular SAM pool, it should be noted that SAH is a very potent non-competitive inhibitor of DNA methylation and only very low concentrations of intracellular SAH may be needed to cause a meaningful inhibition of the enzymatic DNA methylation. Besides, there are many other catechol-containing polyphenolic compounds present in our daily diet, such as tea catechins and bioflavonoids, and they are excellent substrates for human COMT-mediated methylation (13–16). Undoubtedly, the total intake of various dietary catechol-containing polyphenols could be quite large in quantities, which could be a significant cumulative factor that may contribute to the increased intracellular accumulation of SAH, and these dietary chemicals may jointly contribute to the inhibition of DNA methylation in meaningful ways.

Conflict of Interest Statement: None declared.

References


*Received June 2, 2005; revised and accepted July 29, 2005*