



# Dietary Polyphenols May Affect DNA Methylation<sup>1-3</sup>

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## Abstract

Certain dietary polyphenols, such as (-)-epigallocatechin 3-gallate (EGCG) from green tea and genistein from soybean, have been demonstrated to inhibit DNA methyltransferases (DNMT) in vitro. This inhibitory activity is associated with the demethylation of the CpG islands in the promoters and the reactivation of methylation-silenced genes such as *p16<sup>INK4a</sup>*, retinoic acid receptor  $\beta$ , *O*<sup>6</sup>-methylguanine methyltransferase, human *mutL* homolog 1, and glutathione *S*-transferase- $\pi$ . These activities have been observed in human esophageal, colon, prostate, and mammary cancer cell lines, and the activity can be enhanced by the presence of histone deacetylase inhibitors or by a longer-term treatment. Many other polyphenolic compounds have lower activities in inhibiting DNMT. Catechol polyphenols may indirectly inhibit DNMT by generating *S*-adenosyl-L-homocysteine on their methylation by *S*-adenosyl-L-methionine. In theory, prevention or reversal of hypermethylation-induced inactivation of key tumor suppression genes or receptor genes by DNMT inhibitors could be an effective approach for cancer prevention. Because of the rather low bioavailability of most polyphenolic compounds, how much of an effect dietary polyphenols would have on DNA methylation in humans is not clear. The effect of normal dietary consumption of a single polyphenolic compound is probably insignificant. However, the combination of polyphenols with dietary histone deacetylase inhibitors and the additive effect of different dietary chemicals may produce some effects. On the other hand, the consumption of excessive amounts of polyphenols in dietary supplements may affect DNA methylation status. All these possibilities remain to be examined. *J. Nutr.* 137: 223S–228S, 2007.

DNA methylation, primarily at the C5 position of cytosine, affects gene expression in many biological processes such as differentiation, genomic imprinting, DNA mutation, and DNA repair (1–3). DNA hypermethylation, usually occurring at promoter CpG islands, is a major epigenetic mechanism in silencing the expression of genes (4–7). The importance of promoter hypermethylation as well as global hypomethylation in carcinogenesis has been extensively discussed (5–9).

In the past several years, our laboratory has studied DNA hypermethylation during the course of human esophageal carcinogenesis using resected esophageal samples and esophageal

biopsy samples from Linxian (now named Linzhou City), a high esophageal cancer incidence area in northern China (10–14). We found that genes such as *O*<sup>6</sup>-methylguanine methyltransferase (*MGMT*),<sup>4</sup> retinoic acid receptor  $\beta$  (*RAR* $\beta$ ), the tumor suppressor *p16<sup>INK4a</sup>*, and the DNA repair gene *hMLH1* were frequently inactivated by hypermethylation in early lesions of esophageal basal cell hyperplasia. In samples with more advanced lesions, dysplasia, and squamous cell carcinoma, the frequencies of hypermethylation of these genes were even higher, and additional genes were inactivated by DNA hypermethylation. These events, as illustrated in **Figure 1**, together with p53 mutations and Rb aberrations, are believed to contribute to carcinogenesis (10–14). DNA hypermethylation and the inactivation of many of these genes also occurred in esophageal cancer cell lines such as KYSE 510 and KYSE 150; some of these events were reversed by treatment with 2'-deoxy-5-azacytidine (DAC), an inhibitor of 5-cytosine DNA methyltransferase (DNMT).

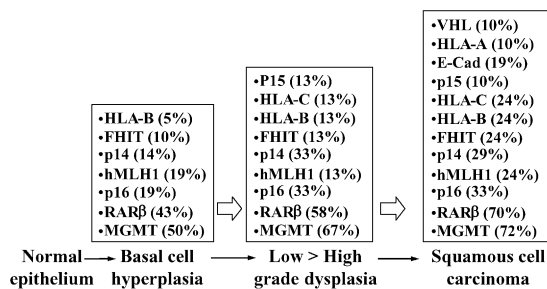
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<sup>4</sup> Abbreviations used: COMT, catechol *O*-methyltransferase; DAC, 2'-deoxy-5-azacytidine; DAPK1, death-associated protein kinase 1; DiMeEGCG, 4',4''-dimethyl EGCG; DNMT, 5-cytosine DNA methyltransferase; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-gallate; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin 3-gallate; ER $\alpha$ , estrogen receptor  $\alpha$ ; GSTP1, glutathione *S*-transferase- $\pi$ ; HDAC, histone deacetylase; hMLH1, human *mutL* homolog 1; MeEGCG, 4''-methyl EGCG; MGMT, *O*<sup>6</sup>-methylguanine methyltransferase; RAR $\beta$ , retinoic acid receptor  $\beta$ ; RASSF1A, Ras association domain family 1A; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosylmethionine; SFN, sulforaphane; TSA, trichostatin.



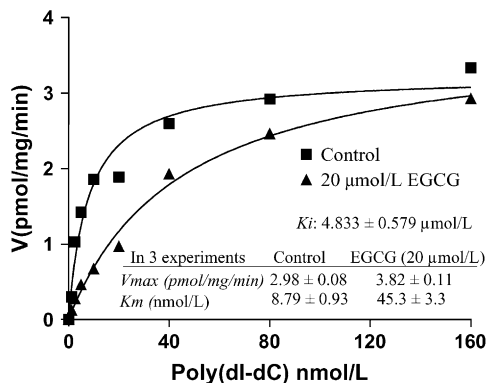
**Figure 1** Promoter hypermethylation of genes in human esophageal squamous carcinogenesis. The frequencies of hypermethylation of specific genes were analyzed in resected and biopsy esophageal samples with different lesions by methylation-specific PCR (10–14).

The development of DNMT inhibitors such as DAC and zebularine as cancer therapeutic agents is a very active research field (8,15,16). In theory, prevention or reversal of hypermethylation-induced inactivation of key tumor suppression genes or receptor genes by DNMT inhibitors could be an effective approach for cancer prevention. Strong inhibitors such as DAC and zebularine may not be suitable for this purpose because of their toxicity. Therefore, we looked into dietary constituents such as (–)-epigallocatechin-3-gallate (EGCG), the most abundant and active polyphenol in green tea, and genistein, a well-studied isoflavone from soy. Previously, Day et al. (17) reported that the consumption of a genistein diet by mice was positively correlated with changes in prostate DNA methylation at CpG islands as determined by differential methylation hybridization.

### Inhibition of catechol O-methyltransferases and DNMT by EGCG and analogs

EGCG has been well studied for its anticancer activities but the mechanisms are not well understood (18–20). In our studies on the biotransformation of tea polyphenols, we found that EGCG is readily methylated by S-adenosylmethionine (SAM) at the 4' and 4'' positions in the presence of catechol O-methyltransferase (COMT) (21–23). Demethylation of SAM results in the formation of S-adenosyl-L-homocysteine (SAH). Through the use of 3,4-dihydroxy-L-phenylalanine and catecholestrogens as substrates for COMT, EGCG was found to be a mixed-type (competitive and noncompetitive) inhibitor (23,24).

Because COMT and DNMT belong to the same superfamily of SAM-dependent methyltransferases with common core structures, we suspected that EGCG may also be an inhibitor of DNMT. With nuclear extracts from KYSE 510 cells as the source of DNMT and poly(dI-dC)·poly(dI-dC) as the substrate, EGCG was found to be a competitive inhibitor of DNMT with a  $K_i$  of 4.8  $\mu\text{mol/L}$  (Fig. 2). [This value is slightly lower than the value reported previously (25). The present  $K_m$ , 8.79 nmol/L, is more accurate than our previous value because of an error in our previous calculation.] EGCG structural analogs from green tea, (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epicatechin (EC) as well as EGCG metabolites 4''-methyl EGCG (MeEGCG) and 4',4''-dimethyl EGCG (DiMeEGCG), all inhibited DNMT dose-dependently: EGCG > ECG, MeEGCG > EGC, and DiMeEGCG > EC (25). Molecular modeling of the interaction between EGCG and DNMT revealed a substantial interactive region with hemimethylated DNA and a cytosine-active pocket for subsequent methylation (25). Docking of EGCG into this pocket indicated that the gallate moiety (D-ring) was oriented at approximately the same position as the



**Figure 2** Kinetic studies on the inhibition of 5-cytosine DNA methyltransferase (DNMT) by (–)-epigallocatechin 3-gallate (EGCG). The reaction mixture, containing 10  $\mu\text{mol/L}$  of S-adenosyl-L-[methyl- $^3\text{H}$ ]methionine and different concentrations of poly(dI-dC)·poly(dI-dC), was incubated for 1.5 h. Each data point represents the mean of 1 duplicate set of incubations, and the data were analyzed by GraphPad Prism 4. The kinetic data were from 3 sets of experiments. In the absence of EGCG, the means  $\pm$  SE of the  $V_{max}$  was 2.98  $\pm$  0.08 pmol/min/mg and of the  $K_m$  was 8.79  $\pm$  0.93 nmol/L. In the presence of 20  $\mu\text{mol/L}$  EGCG, the  $V_{max}$  was 3.82  $\pm$  0.11 pmol/min/mg, and  $K_m$  was 45.3  $\pm$  3.3 nmol/L, showing a competitive inhibition with a  $K_i$  of 4.83  $\pm$  0.58  $\mu\text{mol/L}$ . [These experimental conditions were different from our previous publication (25)].

pyrimidyl ring of cytosine, with possible hydrogen bond formation with Glu<sup>1265</sup> and Pro<sup>1223</sup>, in the structural model of DNMT1. In addition, possible hydrogen bond formation between the hydroxyl groups of the EGCG A and B rings with Ser<sup>1229</sup> and Cys<sup>1225</sup>, respectively, also may have contributed to the high-affinity binding. This model can also explain the lower inhibitory activities of EGCG analogs and metabolites.

### Reversal of hypermethylation and reactivation of RARβ, MGMT, p16<sup>INK4a</sup>, and hMLH1 genes by EGCG

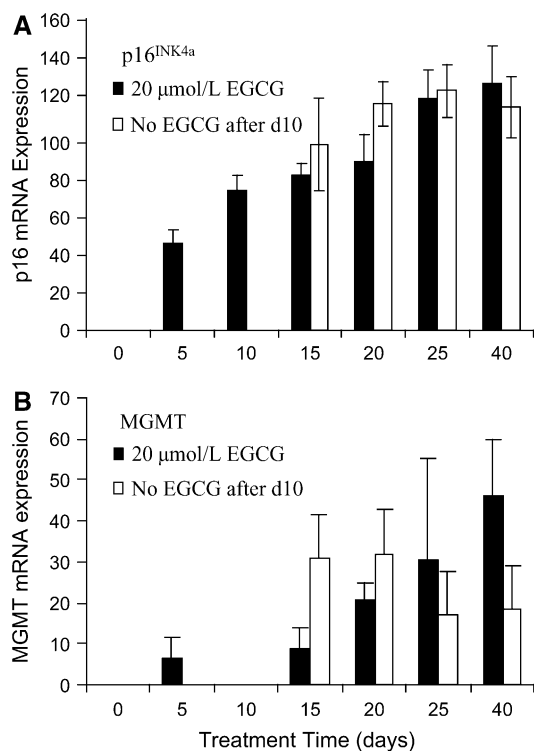
In KYSE 510 cells, p16<sup>INK4a</sup>, RARβ, MGMT, and hMLH1 genes are hypermethylated at the CpG islands in the promoter regions, and the genes are inactivated. The unmethylation-specific bands of these 4 genes appeared after treatment of the cells with 20 or 50  $\mu\text{mol/L}$  of EGCG for 6 d (25). Corresponding to the appearance of the unmethylation-specific bands was the reexpression of mRNA of these genes. The reversal of hypermethylation and reactivation of these genes by EGCG were similar to those produced by the classical DNMT inhibitor DAC. After treatment with 20  $\mu\text{mol/L}$  of EGCG for 48 h, unmethylation-specific bands for these genes began to appear. The mRNA expression of these genes was also observed at 48 h; higher levels were observed at either 72 or 144 h depending on the genes. The reactivation of RARβ and hMLH1 was also demonstrated at the protein level by Western blots. The abilities of EGCG analogs and metabolites to reactivate RARβ mRNA roughly correlated to their inhibitory activities against DNMT (25). We also demonstrated the reactivation of RARβ in esophageal cancer cell line KYSE 150 and prostate cancer cell line PC3 as well as the activation of p16<sup>INK4a</sup> in colon cancer cell line HT-29 after treatment with 20  $\mu\text{mol/L}$  of EGCG for 6 d (25).

The inhibition of DNMT and the partial demethylation of hypermethylated RARβ by EGCG were subsequently demonstrated in breast cancer cell lines MCF-7 and MDA-MB-231 cells (26). Similarly, EGCG or green tea polyphenols treatment was shown to cause the activation of glutathione S-transferase- $\pi$  gene (GSTP1) in prostate cancer LNCaP cells (27,28). However, the effect of EGCG may be gene specific or cell line specific and

was not as robust as DAC (29). Significant demethylation and activation of several genes by EGCG were not observed by Chuang et al. (29) and Stresemann et al. (30). Mittal et al. (31) reported that topical applications of EGCG to the mouse skin inhibited UVB-induced global DNA hypomethylation. Because global DNA hypomethylation has been reported to be associated with hypermethylation and inactivation of specific genes during carcinogenesis (9), this observation is not necessarily contradictory to the concept that EGCG can prevent or reverse the hypermethylation of certain specific genes.

### Reactivation of *p16<sup>INK4a</sup>* and *MGMT* by long-term treatment with EGCG and by its combination with other agents

To determine whether long-term treatment of cells with EGCG can increase the extent of reactivation of methylation-silenced genes, we treated KYSE 510 cells with EGCG for different lengths of time. EGCG (20  $\mu\text{mol/L}$ ) was administered to the cells in fresh cell culture medium every other day. As shown in Figure 3, the *p16<sup>INK4a</sup>* mRNA level increased with time; on days 25 and 40 the levels were  $\sim 3$  times that of day 5. Withdrawing EGCG at day 10 did not seem to affect the subsequent increase of *p16<sup>INK4a</sup>* mRNA. The pattern of changes of the *MGMT* mRNA levels was not as clear even though a time-dependent increase was observed on continuous treatment with EGCG. After EGCG treatment was withdrawn on day 10, the *MGMT* mRNA levels seemed to increase on days 15 and 20 but to decrease on days 25 and 40. In these long-term treatment studies, the unmethylation-



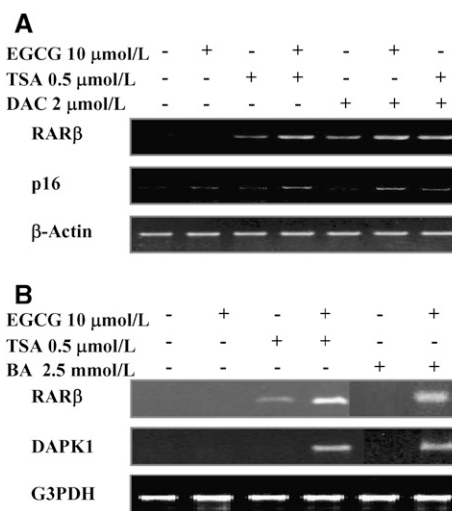
**Figure 3** Effect of long-term EGCG treatment on reexpression of *p16<sup>INK4a</sup>* (A) and *O*<sup>6</sup>-methylguanine methyltransferase (*MGMT*, B) mRNA. KYSE 510 cells were subcultured every 5 d and treated with 20  $\mu\text{mol/L}$  EGCG every other day when changing medium (■). In another group, EGCG was withdrawn after day 10 (□). mRNA levels were determined with RT-PCR, and the band intensity was quantified using densitometry and normalized to each endogenous control,  $\beta$ -actin. The results are expressed as means  $\pm$  SE from 3 independent experiments.

specific bands of *p16<sup>INK4a</sup>* and *MGMT* genes also appeared to increase with time (data not shown). These results are preliminary; additional studies are needed to confirm these results.

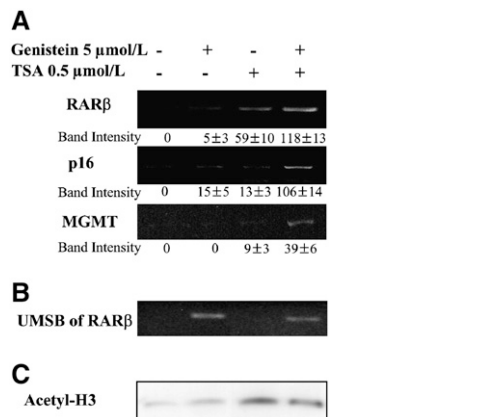
Treatment of KYSE 510 cells with 10  $\mu\text{mol/L}$  of EGCG only weakly activated *RAR $\beta$*  and *p16<sup>INK4a</sup>*, showing faint mRNA bands as determined by RT-PCR. Treatment with 0.5  $\mu\text{mol/L}$  of trichostatin (TSA), a histone deacetylase (HDAC) inhibitor, also slightly activated these genes (Fig. 4 A). The combination of EGCG with TSA appeared to synergistically increase mRNA levels. The combination of DAC (2  $\mu\text{mol/L}$ ) with EGCG (10  $\mu\text{mol/L}$ ) or TSA (0.5  $\mu\text{mol/L}$ ) also produced a synergistic or additive effect. Figure 4B shows the apparent synergistic action between EGCG (10  $\mu\text{mol/L}$ ) and TSA (0.5  $\mu\text{mol/L}$ ) or butyric acid (2.5 mmol/L) in the reactivation of *RAR $\beta$*  and the gene for death-associated protein kinase 1 (*DAPK1*) in mouse lung cancer cell line CL13. The nature of these actions remains to be characterized further.

### Reversal of hypermethylation and reactivation of *p16<sup>INK4a</sup>*, *RAR $\beta$* , and *MGMT* by genistein and other compounds

Treatment of KYSE 510 cells with genistein (2–20  $\mu\text{mol/L}$ ) partially reversed DNA hypermethylation and reactivated *p16<sup>INK4a</sup>*, *RAR $\beta$* , and *MGMT* (32). This was indicated by the appearance of unmethylation-specific bands by methylation-specific PCR as well as by the increased mRNA levels as determined by RT-PCR and real-time PCR. Partial reversal of DNA hypermethylation and reactivation of *RAR $\beta$*  by genistein were also observed in KYSE 150 cells and prostate cancer LNCaP and PC3 cells. Genistein (20–50  $\mu\text{mol/L}$ ) dose-dependently inhibited DNMT activity, showing competitive and noncompetitive inhibition with respect to the substrate poly(dI-dC)-poly(dI-dC) and noncompetitive inhibition with respect to SAM (32). Two other isoflavones, biochanin A and daidzein, were less effective in



**Figure 4** Effects of combination of EGCG with trichostatin (TSA), 2'-deoxy-5-azacytidine (DAC), or butyric acid (BA) on the reactivation of methylation-silenced genes. (A) KYSE 510 cells were treated with 10  $\mu\text{mol/L}$  EGCG, 2  $\mu\text{mol/L}$  DAC, or both for 5 d and cultured for 1 additional day in fresh medium with or without 0.5  $\mu\text{mol/L}$  TSA. (B) CL13 cells [established from NNK-induced mouse lung tumor, obtained from Dr. S. Belinski (39)] were treated with 10  $\mu\text{mol/L}$  EGCG every day for 5 d and cultured for 1 additional day in fresh medium with 0.5  $\mu\text{mol/L}$  TSA or 2.5 nmol/L BA. The mRNA expression levels were determined by RT-PCR.  $\beta$ -Actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used as the endogenous control. The results were reproduced in another set of experiments.



**Figure 5** Effects of combination of genistein and TSA on epigenetic changes. KYSE 510 cells were treated with 5 μmol/L genistein for 5 d and cultured for 1 additional day in fresh medium with 0.5 μmol/L TSA. (A) mRNA levels were determined with RT-PCR, and the band intensity was quantified using densitometry and normalized to each endogenous control (means ± SE,  $n = 2$ ). (B) Unmethylated-specific DNA levels of *RARβ* were determined with methylation-specific PCR. (C) Acetylated histone 3 (H3) levels were determined with a Western blot. The results were reproduced in another set of experiments.

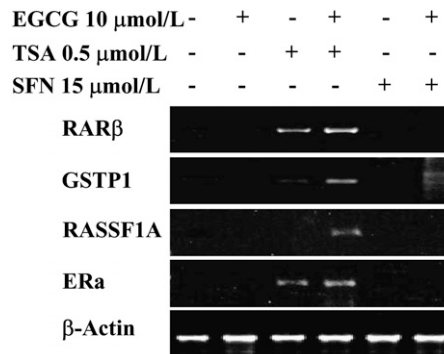
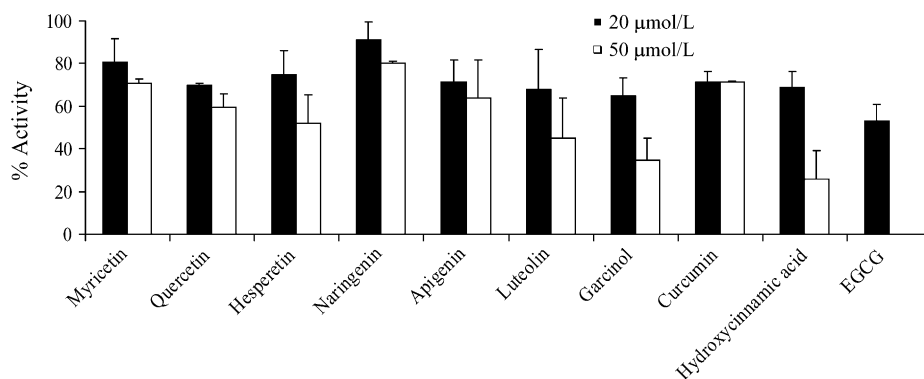
inhibiting DNMT activity, reactivating *RARβ*, and inhibiting cancer cell growth. Genistein was a weaker DNMT inhibitor than EGCG, yet it was just as active or more active in demethylating hypermethylated genes and reactivating their expression. One possible reason for this is that genistein is more stable than EGCG in the cell culture medium and reaches higher intracellular concentrations than EGCG (data not shown). Another possibility is that genistein is also an (albeit weak) inhibitor of HDAC (32).

Treatment of KYSE 510 cells with a combination of genistein (5 μmol/L) with TSA (0.5 μmol/L) synergistically increased the mRNA levels of p16<sup>INK4a</sup>, *RARβ*, and *MGMT*. The level of unmethylation-specific DNA band of *RARβ* was increased by genistein but not by TSA (Fig. 5). The level of acetylated H3 was increased by TSA but not by genistein. In LNCaP cells, TSA significantly enhanced the effectiveness of genistein in reactivating *RARβ*, *GSTP1*, the gene for the Ras association domain family 1A (*RASSF1A*), and the gene for estrogen receptor α (*ERα*), whereas sulforaphane (SFN, 15 μmol/L) only slightly enhanced the reactivation of *GSTP1* (Fig. 6).

#### Inhibition of DNMT activity by other polyphenols

In addition to the aforementioned polyphenols and isoflavones, we also studied some other commonly used phenolic compounds to determine their effects on the DNMT activity in nuclear extracts of KYSE 510 cells (Fig. 7). These include myricetin and

**Figure 7** Inhibition of 5-cytosine DNA methyltransferase activity by different polyphenols. The reaction mixture contained nuclear extracts (4.5 μg protein), poly(dI-dC)-poly(dI-dC) (0.75 μg; 20 nmol/L), and S-adenosyl-L-[methyl-<sup>3</sup>H]methionine (10 μmol/L, 2.0 μCi) in a 40-μL incubation mixture containing 10% glycerol and 2 mmol/L 2-mercaptoethanol. The incubation time was 1.5 h. Each data point represents the mean of a set of duplicate analyses. The Y-error bars reflect the difference of the duplicate.



**Figure 6** Effects of the combination of genistein with TSA or sulforaphane (SFN) on reactivation of methylation-silenced genes. LNCaP cells were treated with 10 μmol/L genistein alone for 5 d and cultured for 1 additional day in fresh medium with 0.5 μmol/L TSA or 15 μmol/L SFN. mRNA levels of these genes were determined with RT-PCR. The results were reproduced in another set of experiments.

quercetin (flavanols), hesperetin and naringenin (flavanols), apigenin and luteolin (flavanols), garcinol, curcumin, and hydroxycinnamic acid. All these compounds inhibited DNMT activities at 20 and 50 μmol/L, but their activities were lower than that of EGCG. At 50 μmol/L, hydroxycinnamic acid, garcinol, and luteolin inhibited DNMT activity by >50%.

Compounds possessing catechol structures are readily methylated by SAM in the presence of COMT, resulting in the conversion of SAM to SAH. The SAM:SAH ratio could affect DNMT activity. Lee et al. (26,33) demonstrated that many catechol polyphenols, such as quercetin, fisetin, and myricetin, inhibited DNMT by converting SAM to SAH and that myricetin is a strong inhibitor of DNMT. This mechanism was also proposed for the partial reversal of the promoter hypermethylation of the *RARβ* in breast cancer cell lines by caffeic acid and chlorogenic acid (33).

#### Alteration of levels of SAM, SAH, and homocysteine after the administration of EGCG to mice

Consumption of polyphenols was reported to reduce SAM levels and increase homocysteine levels in humans (34). A severe decrease in SAM or the accumulation of SAH could reduce DNMT activity. We have examined this issue in our ongoing experiments on bioavailability, toxicity, and cancer-preventive activities of EGCG. The results are summarized as follows: 1) Administration of 0.16% or 0.32% EGCG in drinking fluid to CF-1 mice for 7 d had no significant effect on plasma homocysteine or methionine levels. 2) Administration of 0.5% Polyphenon E (a standardized green tea polyphenol preparation containing 65% EGCG and 15% other catechins) in drinking fluid to A/J mice for 7 d

**TABLE 1** Effects of (–)-epigallocatechin 3-gallate (EGCG) treatment on tissue levels of S-adenosylmethionine (SAM) and S-adenosyl-L-homocysteine (SAH)<sup>1</sup>

Dose, mg/kg	Liver			Small Intestine		
	SAH, nmol/g	SAM, nmol/g	SAM/SAH	SAH, nmol/g	SAM, nmol/g	SAM/SAH
0	44.1 ± 3.3	58.2 ± 1.1	1.3 ± 0.1	4.1 ± 0.4	71.4 ± 3.9	17.8 ± 1.6
50	43.2 ± 6.7	57.0 ± 6.2	1.3 ± 0.2	3.9 ± 0.8	73.6 ± 6.0	18.6 ± 2.5
100	44.1 ± 3.9	44.9 ± 1.7	1.0 ± 0.2	2.6 ± 0.2*	70.7 ± 5.2	26.9 ± 1.5*
200	32.3 ± 2.3*	27.9 ± 0.8*	0.9 ± 0.1	2.2 ± 0.2*	72.1 ± 6.7	33.5 ± 1.9*
500	33.0 ± 2.6*	22.2 ± 2.3*	0.6 ± 0.1*	3.8 ± 0.4	68.3 ± 6.0	17.9 ± 2.0
1000	29.2 ± 1.8*	16.2 ± 0.9*	0.6 ± 0.1*	3.7 ± 0.3	67.0 ± 6.3	18.0 ± 1.6
2000	31.4 ± 2.4*	9.4 ± 1.0*	0.3 ± 0.1*	3.6 ± 0.4	58.6 ± 6.2	16.2 ± 1.5

<sup>1</sup> CF1 mice were administered i.g. a single dose of EGCG (50–2000 mg/kg). After 3 h, liver and small intestine were removed. The levels of S-adenosylmethionine (SAM) and S-adenosyl-L-homocysteine (SAH) were analyzed by HPLC. Data are presented as means ± SE (*n* = 6).

\* *P* < 0.05.

decreased the small intestinal level of SAM (34.5 vs. 45.0 nmol/g in the control group) without altering SAH level (16–19 nmol/g). The treatment did not affect the hepatic SAM and SAH levels. 3) Administration of 0.16% EGCG in drinking fluid to *Apc<sup>min/+</sup>* mice for 8 or 9 wk significantly decreased the small intestinal SAM levels (40.5 vs. 58.2 nmol/g in the control group) without affecting the SAH level. The treatment did not affect SAM and SAH levels in liver. EGCG (0.08%), administered similarly in drinking fluid, had no effect on small intestinal or hepatic levels of SAM and SAH. 4) Treatment of male CF-1 mice with a single i.g. dose of EGCG at 2000 mg/kg significantly elevated plasma levels of homocysteine at 50 min and 3 h after treatment (both with ~6–7 μmol/L vs. 3.9 μmol/L in the control group). At 8 h after the treatment, the plasma levels of homocysteine and methionine partially returned to but still significantly differed from the basal level. The hepatic SAM level decreased from a basal level of 60.9 nmol/g to 10.4, 11.4, and 41.2 nmol/g at 30 min, 3 h, and 8 h, respectively. The corresponding levels of SAH also decreased from 44.0 nmol/g to 32.9, 34.6, and 36.1 nmol/g, respectively. Apparently the SAH was converted to homocysteine. 5) Results from a dose-response study with i.g. administration of EGCG at 50–2000 mg/kg to male CF-1 mice are summarized in Table 1. At 3 h after the treatment, the hepatic SAM and SAH levels as well as the SAM:SAH ratio significantly decreased with higher concentrations of EGCG. With EGCG at 50 mg/kg, the SAH levels appeared to be higher than the basal level, but the difference was not statistically significant.

These studies indicate that the administration of EGCG (or Polyphenon E) through drinking fluid can moderately decrease the level of SAM (without increasing the level of SAH) in the intestine but have no significant effect on hepatic SAM or SAH levels. Acute i.g. treatment with high doses of EGCG significantly elevated plasma levels of homocysteine, decreased the levels of plasma methionine, and decreased the levels of intestinal SAM and SAH. A calculation based on caloric consumption indicates that a dose of 1000 mg/kg is equivalent to the consumption of ~4200 mg of EGCG or 20–35 cups of green tea by an individual, and it may produce toxicity.

### Discussion and conclusions

The discussed studies demonstrate the inhibition of DNMT and promoter cytosine hypermethylation as well as the reactivation of some methylation-silenced genes by EGCG, genistein, and related compounds. Although the activation of methylation-silenced genes in cell lines by the compounds appears to correlate with the inhibition of DNMT enzyme activity in nuclear extracts,

other mechanisms of actions, such as the inhibition of HDAC, may also play a role. The effective concentrations of EGCG (10–50 μmol/L) observed in studies with cell lines are ~50 times higher than the plasma and tissue levels of EGCG generally observed after ingestion of tea (35,36). The oral digestive tract has direct contact and may be exposed to higher levels of EGCG. The effective genistein concentrations observed (5–20 μmol/L) for DNA demethylation are close to but still higher than the plasma level of genistein (0.7–6.0 μmol/L) reported after consumption of soy products by women (37,38). Whether EGCG, genistein, and other dietary polyphenols can reverse DNA hypermethylation and reactivate methylation-silenced genes *in vivo* still remain to be determined. A key issue is whether long-term treatment of cells or animals with these agents can increase their efficacies; this subject deserves further investigation.

The possibility that catechol polyphenols may inhibit DNMT indirectly by decreasing SAM and increasing SAH is very interesting. The key element of this hypothesis is that SAH is a potent inhibitor of DNMT (33). Our studies, however, suggest that this type of inhibition may not occur *in vivo* because elevated tissue levels of SAH were not observed and only a modest decrease in SAM levels was observed after oral administration of EGCG. Only high acute doses of EGCG cause a marked decrease of SAM (and SAH) levels in liver; this may be a situation associated with toxicity.

Consumption of polyphenols from diet and beverages will not have a major effect on DNA methylation in animals and humans with a normal diet. The effect may be more significant, however, if the diet has limited amounts of methyl donors. With the consumption of large quantities of polyphenols through dietary supplements, the high tissue level of polyphenols may affect DNA methylation. This possibility remains to be demonstrated. This may also be a situation where caution needs to be applied to avoid possible toxicity from the oxidation of polyphenols. For the prevention of cancer, the combination of polyphenol DNMT inhibitors together with HDAC inhibitors, such as butyric acid and sulforaphane, or other inhibitors are promising approaches.

### Literature Cited

1. Ehrlich M. DNA methylation in cancer: too much, but also too little. *Oncogene*. 2002;21:5400–13.
2. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet*. 1999;21:163–7.

3. Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis*. 1997;18:869–82.
4. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*. 2003;349:2042–54.
5. Jones PA. DNA methylation and cancer. *Oncogene*. 2002;21:5358–60.
6. Bird AP. CpG-rich islands and the function of DNA methylation. *Nature*. 1986;321:209–13.
7. Antequera F, Bird A. CpG islands. *EXS*. 1993;64:169–85.
8. Laird PW. Cancer epigenetics. *Hum Mol Genet*. 2005;14:R65–76.
9. Robertson KD. DNA methylation and human disease. *Nat Rev Genet*. 2005;6:597–610.
10. Xing EP, Nie Y, Wang LD, Yang GY, Yang CS. Aberrant methylation of p16INK4a and deletion of p15INK4b are frequent events in human esophageal cancer in Linxian, China. *Carcinogenesis*. 1999;20:77–84.
11. Xing EP, Yang GY, Wang LD, Shi ST, Yang CS. Loss of heterozygosity of the Rb gene correlates with pRb protein expression and associates with p53 alteration in human esophageal cancer. *Clin Cancer Res*. 1999;5:1231–40.
12. Nie Y, Liao J, Zhao X, Song Y, Yang GY, Wang LD, Yang CS. Detection of multiple gene hypermethylation in the development of esophageal squamous cell carcinoma. *Carcinogenesis*. 2002;23:1713–20.
13. Wang Y, Fang MZ, Liao J, Yang GY, Nie Y, Song Y, So C, Xu X, Wang LD, Yang CS. Hypermethylation-associated inactivation of retinoic acid receptor beta in human esophageal squamous cell carcinoma. *Clin Cancer Res*. 2003;9:5257–63.
14. Fang MZ, Jin Z, Wang Y, Liao J, Yang GY, Wang LD, Yang CS. Promoter hypermethylation and inactivation of O(6)-methylguanine-DNA methyltransferase in esophageal squamous cell carcinomas and its reactivation in cell lines. *Int J Oncol*. 2005;26:615–22.
15. Issa JP. Optimizing therapy with methylation inhibitors in myelodysplastic syndromes: dose, duration, and patient selection. *Nat Clin Pract Oncol*. 2005;2: Suppl 1:S24–9.
16. Silverman LR, Mufti GJ. Methylation inhibitor therapy in the treatment of myelodysplastic syndrome. *Nat Clin Pract Oncol*. 2005;2: Suppl 1: S12–23.
17. Day JK, Bauer AM, DesBordes C, Zhuang Y, Kim BE, Newton LG, Nehra V, Forsee KM, MacDonald RS, et al. Genistein alters methylation patterns in mice. *J Nutr*. 2002;132: 8 Suppl:2419S–23S.
18. Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. *Annu Rev Pharmacol Toxicol*. 2002;42:25–54.
19. Lambert JD, Hong J, Yang GY, Liao J, Yang CS. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. *Am J Clin Nutr*. 2005;81: 1 Suppl:284S–91S.
20. Yang CS, Lambert JD, Hou Z, Ju J, Lu G, Hao X. Molecular targets for the cancer preventive activity of tea polyphenols. *Mol Carcinog*. 2006; 45:431–5.
21. Meng X, Lee MJ, Li C, Sheng S, Zhu N, Sang S, Ho CT, Yang CS. Formation and identification of 4'-O-methyl(-)-epigallocatechin in humans. *Drug Metab Dispos*. 2001;29:789–93.
22. Meng X, Sang S, Zhu N, Lu H, Sheng S, Lee MJ, Ho CT, Yang CS. Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. *Chem Res Toxicol*. 2002;15:1042–50.
23. Lu H, Meng X, Yang CS. Enzymology of methylation of tea catechins and inhibition of catechol-O-methyltransferase by (-)-epigallocatechin gallate. *Drug Metab Dispos*. 2003;31:572–9.
24. Chen D, Wang CY, Lambert JD, Ai N, Welsh WJ, Yang CS. Inhibition of human liver catechol-O-methyltransferase by tea catechins and their metabolites: structure-activity relationship and molecular-modeling studies. *Biochem Pharmacol*. 2005;69:1523–31.
25. Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W, Yang CS. Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res*. 2003;63:7563–70.
26. Lee WJ, Shim JY, Zhu BT. Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids. *Mol Pharmacol*. 2005;68:1018–30.
27. Shukla S, Trokhan S, Resnick MI, Gupta S. Epigallocatechin-3-gallate causes demethylation and activation of GSTP1 gene expression in human prostate cancer LNCaP cells. *Proc Amer Assoc Cancer Res*. 2005;46:1572.
28. Pandey M, Gupta S. Green tea polyphenols (GTP) cause activation of the GSTP1 gene in prostate cancer LNCaP cells by promoter demethylation. *Proc Amer Assoc Cancer Res*. 2006;47:1614.
29. Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS, Jones PA. Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. *Mol Cancer Ther*. 2005;4: 1515–20.
30. Stressemann C, Brueckner B, Musch T, Stopper H, Lyko F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res*. 2006;66:2794–800.
31. Mittal A, Piyathilake C, Hara Y, Katiyar SK. Exceptionally high protection of photocarcinogenesis by topical application of (-)-epigallocatechin-3-gallate in hydrophilic cream in SKH-1 hairless mouse model: relationship to inhibition of UVB-induced global DNA hypomethylation. *Neoplasia*. 2003;5:555–65.
32. Fang MZ, Chen D, Sun Y, Jin Z, Christman JK, Yang CS. Reversal of hypermethylation and reactivation of p16INK4a, RARbeta, and MGMT genes by genistein and other isoflavones from soy. *Clin Cancer Res*. 2005;11:7033–41.
33. Lee WJ, Zhu BT. Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols. *Carcinogenesis*. 2006;27:269–77.
34. Olthof MR, Hollman PC, Zock PL, Katan MB. Consumption of high doses of chlorogenic acid, present in coffee, or of black tea increases plasma total homocysteine concentrations in humans. *Am J Clin Nutr*. 2001;73:532–8.
35. Lee MJ, Maliakal P, Chen L, Meng X, Bondoc FY, Prabhu S, Lambert G, Mohr S, Yang CS. Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiol Biomarkers Prev*. 2002;11:1025–32.
36. Lambert JD, Lee MJ, Diamond L, Ju J, Hong J, Bose M, Newmark HL, Yang CS. Dose-dependent levels of epigallocatechin-3-gallate in human colon cancer cells and mouse plasma and tissues. *Drug Metab Dispos*. 2006;34:8–11.
37. Adlercreutz CH, Goldin BR, Gorbach SL, Hockerstedt KA, Watanabe S, Hamalainen EK, Markkanen MH, Makela TH, Wahala KT, Adlercreutz T. Soybean phytoestrogen intake and cancer risk. *J Nutr*. 1995;125: 3 Suppl:757S–70S.
38. Xu X, Harris KS, Wang HJ, Murphy PA, Hendrich S. Bioavailability of soybean isoflavones depends upon gut microflora in women. *J Nutr*. 1995;125:2307–15.
39. Wardlaw SA, Zhang N, Belinsky SA. Transcriptional regulation of basal cyclooxygenase-2 expression in murine lung tumor-derived cell lines by CCAAT/enhancer-binding protein and activating transcription factor/cAMP response element-binding protein. *Mol Pharmacol*. 2002;62: 326–33.