Chapter 6

Epigenetics, Plant (Poly)phenolics, and Cancer Prevention

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Abstract: Epigenetics describes a phenomenon of modifications in gene expression caused by heritable, but potentially reversible, alterations in the chromatin structure, DNA methylation, and post-transcriptional effects of small noncoding (micro) RNAs (miRNAs), without changes in the DNA sequence. These mechanisms represent novel targets for polyphenols and other natural products in the prevention and treatment of cancer and other diseases. This chapter summarizes the influence of various classes of polyphenol on the enzymatic activities of major enzymes involved in epigenetic gene regulation: DNA and histone methyltransferases (DNMTs and HMTs), histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone demethylases (HDMs). Inhibitory effects mediated by (−)-epigallocatechin 3-gallate (ECGC) from green tea and the isoflavone genistein from soy *in vitro* and *in vivo* are described in detail. Functional consequences on gene expression in cell culture, in rodent models and in human intervention studies, are highlighted.

Keywords: gene regulation, DNA methylation, histone modifications, DNA methyltransferase, epigenome, flavonoids

6.1 Introduction

Genetic information is stored in the cell nucleus in the form of the deoxyribonucleic acid (DNA) sequence, which is organized as a double-stranded molecule consisting of two complementary strands of DNA. All individual cells in an organism contain an identical DNA sequence. Yet mammals possess over 200 different tissue types, which are characterized by unique gene-expression patterns established during development. These

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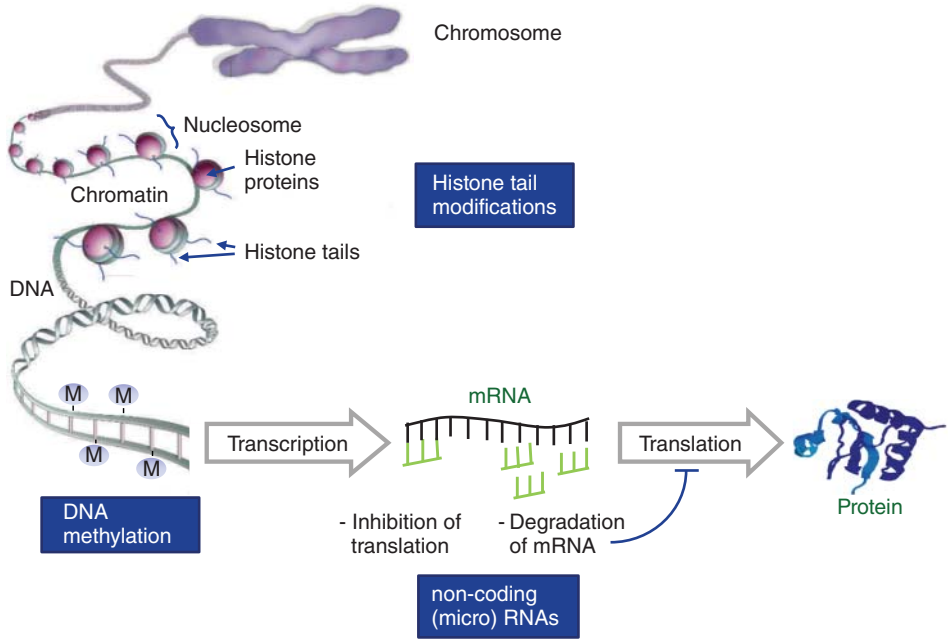


Fig. 6.1 Overview of chromatin organization and epigenetic mechanisms, including DNA methylation, histone modifications, and noncoding miRNAs, targeting DNA, N-terminal histone tails, and mRNA. (Adapted from Qiu, 2006. Reproduced with permission of Nature Publishing Group.) See text for details. For color details, please see Plate 6.1.

fine-tuned differences in gene-expression patterns are maintained by "epigenetics"; that is, modifications in gene expression caused by heritable, but potentially reversible, alterations in chromatin structure and/or DNA methylation, without changes in the DNA sequence (Henikoff & Matzke, 1997). An important level of regulation is embedded in the temporal and spatial accessibility of genomic regions controlled by the chromatin structure. As summarized in Fig. 6.1, chromatin is a higher-order genomic structure, made up of DNA and proteins. The DNA helix is wrapped around pairs of the core histones H2A, H2B, H3, and H4, forming bead-like structures called nucleosomes. For genomic organization, nucleosomes are arranged on the DNA like "beads on a string", which are then folded to form higher-organized chromatin structures. DNA accessibility is regulated by the degree of chromatin condensation. *Heterochromatin* is highly condensed and contains mainly repetitive sequences, whereas *euchromatin* represents genomic regions that are decondensed and contain actively transcribed genes (Editors, 2003). Epigenetic mechanisms that modulate chromatin accessibility include DNA methylation and post-translational modifications of histone tails, such as acetylation, phosphorylation, and methylation. In addition, small noncoding (micro) RNAs (miRNAs) regulate gene expression at the level of translation by degrading mRNA or inhibiting its translation. These mechanisms represent novel targets for polyphenols and other natural products in the prevention and treatment of cancer and other diseases.

6.2 Influence of polyphenols on DNA methylation

6.2.1 DNA methylation in normal and tumor cells

DNA methylation is a post-replication modification of DNA catalyzed by DNA methyltransferases DNMT1, 3a, and 3b that transfer methyl groups from S-adenosyl-*L*-methionine (SAM) as a methyl donor to cytosines, creating 5-methylcytosine (5mC), mainly within the context of CpG dinucleotides (Editors, 2003). DNMT1 is a maintenance methyltransferase that acts on hemimethylated DNA after replication and thus assures transmission of DNA methylation patterns to daughter cells. DNMT3a and DNMT3b are *de novo* methyltransferases that transfer methyl groups to previously unmethylated DNA sequences. DNMT3b is believed to play an important role during tumorigenesis (Beaulieu *et al.*, 2002; Linhart *et al.*, 2007).

In normal cells, CpG-rich sequences (so-called CpG islands, CGIs) in gene-promoter regions are generally unmethylated, with the exception of about 6–8%, which are methylated in a tissue-specific manner (Illingworth *et al.*, 2008). Conversely, the majority of CpG sites in repetitive genomic sequences are heavily methylated, limiting their accessibility and contributing to chromosomal stability (Esteller, 2007). This controlled pattern of DNA methylation is disrupted during aging and the development of chronic diseases. During carcinogenesis, increased methylation (DNA hypermethylation) of promoter CGIs contributes to transcriptional gene silencing, for example of tumor-suppressor genes (Kopelovich *et al.*, 2003; Esteller, 2007; Jones & Baylin, 2007). On the other hand, carcinogenesis is associated with a global loss of DNA methylation at repetitive genomic sequences (DNA hypomethylation), leading to genomic instability and chromosomal aberrations (Gama-Sosa *et al.*, 1983; Goelz *et al.*, 1985) (Fig. 6.2). Importantly, genes silenced by changes in DNA methylation remain intact and can potentially be reactivated by small molecules that affect the activity or expression of DNMTs. Consequently, the development of agents or food components that prevent or reverse cancer-induced DNA methylation and the consequent inactivation of gene expression is a promising new approach in cancer prevention (Huang *et al.*, 2011; Singh *et al.*, 2013).

6.2.2 Inhibition of DNMTs in vitro

Polyphenols are a heterogeneous group of compounds that can be broadly divided into flavonoids, non-flavonoid polyphenols, and phenolic acids (for an overview, see Malireddy *et al.*, 2012). Research on the potential of polyphenols and other natural products to inhibit DNMTs and modulate aberrant DNA methylation was initiated in 2003, when Yang and coworkers first described the DNMT inhibitory activity of the green tea catechin (−)-epigallocatechin 3-gallate (EGCG) (Fang *et al.*, 2003). Since then, various flavonoids and phenolic compounds have been tested in several studies for their potential to inhibit the enzymatic activity of human or bacterial DNMT *in vitro* (Lee *et al.*, 2005; Lee & Zhu, 2006; Fang *et al.*, 2007; Paluszczak *et al.*, 2010; Rajavelu *et al.*, 2011). Table 6.1 gives an overview of the DNMT inhibitory effects of polyphenols, including information on chemical structures and dietary sources. Concentrations tested, percentage of inhibition,

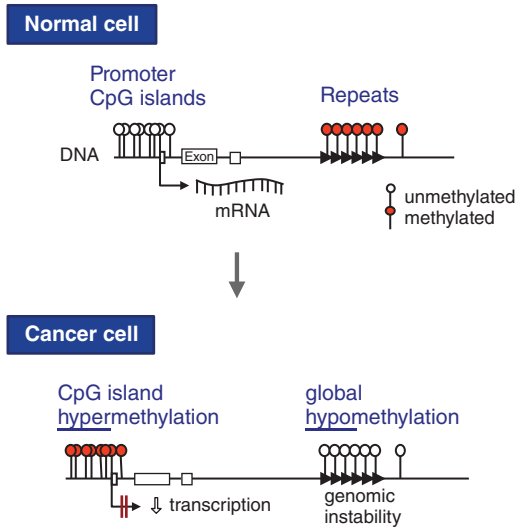


Fig. 6.2 Overview of DNA methylation changes during carcinogenesis, including gain of methylation (red circles) at CpG islands (CGIs), often located in gene promoter regions (CGI hypermethylation) and associated with epigenetic silencing of gene transcription and global loss of DNA methylation (DNA hypomethylation, open circles) at intergenic regions and repetitive sequences, contributing to genomic instability. For color details, please see Plate 6.2.

and half-maximal inhibitory concentrations (IC_{50}) are reported when available. Test systems are based on radioactive detection or an ELISA-based approach, and depending on the assay conditions (human or bacterial enzyme, nuclear extracts or recombinant enzymes as enzyme source, choice of substrate, incubation time, buffer composition, mode of detection), results from these *in vitro* studies may not always be comparable or reproducible (Gilbert & Liu, 2010).

Flavonoid polyphenols tested include flavones, flavonols, flavanones, flavanols, and anthocyanidins. Among the flavones tested, baicalein from *Scrutellaria baicalensis* inhibited DNMT activity by 80% at a concentration of 20 μM and was more potent than apigenin and luteolin. Galangin from propolis or galangal root was identified as the most potent DNMT-inhibitory flavonol, with 67% inhibition at 20 μM. Quercetin, myricetin, and fisetin were less active. Between the two flavanones tested, hesperetin was found to be more active that naringenin, with 49% inhibition at 50 μM. The anthocyanidin cyanidin demonstrated high inhibitory potential, with 94% inhibition at a test concentration of 20 μM.

For polyphenols bearing a catechol moiety, studies by Lee and coworkers (Lee *et al.*, 2005; Lee & Zhu, 2006) suggest an alternative indirect mechanism of DNMT inhibition. These polyphenols may serve as substrates for methylation by catechol-*O*-methyltransferase (COMT), leading to the conversion of S-adenosyl-*L*-methionine (SAM) to S-adenosyl-*L*-homocysteine (SAH), which has been identified as a negative-feedback regulator of methyltransferases, including DNMTs and COMT. On the other hand, catechol-methylation by COMT may lead to depletion of SAM. In the presence of COMT,

catechol-polyphenols inhibit bacterial M.Sss1 or human DNMT activity with IC_{50} values in the range 0.75–8.40 μM.

Non-flavonoid polyphenols tested for DNMT inhibitory potential include the dihydrochalcone phloretin, garcinol from *Garcinia indica* (see Table 6.3), ellagic acid, and stilbenes. Rosmarinic acid was identified as the most potent phenolic acid with DNMT inhibitory potential, with 74% inhibition at a test concentration of 20 μM, and 90% at 40 μM.

6.2.3 Inhibition of DNA methylation in cellular systems and in vivo

As *in vitro* inhibition of enzymatic activities by polyphenols could result from unspecific protein binding, especially at elevated concentrations of inhibitors, demonstration of the functional consequences of DNMT inhibition might be a more reliable approach to the identification of polyphenols targeting the epigenetic mechanisms, including the methylome. As summarized in Table 6.2, quercetin, nordihydroguaiaretic acid (NDGA), resveratrol, and complex extracts of apple and black raspberry polyphenols have been reported to influence DNA methylation in cell culture or *in vivo*.

6.2.3.1 Quercetin

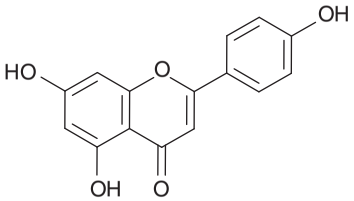
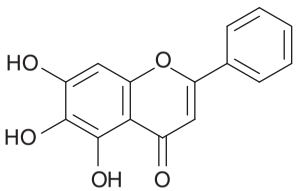
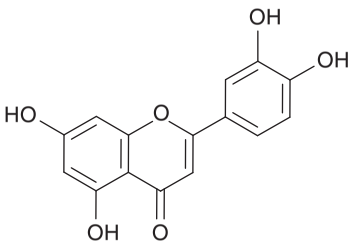
Quercetin is a cancer-chemopreventive flavonoid occurring in the form of its glycosides ubiquitously in fruits, vegetables, and beverages (Russo *et al.*, 2012). It has a broad spectrum of cancer-preventive activities: it acts as an antioxidant and modulates enzymes and signaling cascades involved in detoxification, inflammation, proliferation, apoptosis, angiogenesis, autophagy, immune defense, and senescence (for a summary, see Murakami *et al.*, 2008; Vargas & Burd, 2010; Russo *et al.*, 2012). Cancer-preventive efficacy has been demonstrated in several rodent models for colon, mammary-gland, skin, and lung cancer (Murakami *et al.*, 2008). In addition, studies in human subjects indicate that quercetin intervention reduces markers of oxidative stress and inflammation (Russo *et al.*, 2012).

Beside its potential to inhibit DNMT activity *in vitro*, quercetin treatment of a colon-cancer cell line led to dose-dependent demethylation of the cell cycle-regulator $p16^{INK4A}$ (inhibitor of cyclin-dependent kinase CDK4, also known as CDKN2, CDK inhibitor 2). This was associated with p16 up-regulation at the mRNA and protein level and inhibition of cell proliferation (Tan *et al.*, 2009).

6.2.3.2 Nordihydroguaiaretic acid (NDGA)

NDGA belongs to the naturally occurring plant phenolic lignans and is found at high concentrations in the Creosote bush (*Larrea tridentata* (Sesse and Moc. ex DC) Coville). It is a potent antioxidant and inhibits lipoxygenases and receptor tyrosine kinases such as insulin-like growth factor receptor 1 (IGF-1R). This is associated with tumor-cell growth inhibition *in vitro* and *in vivo*. Chemopreventive efficacy has been demonstrated in UVB- and chemically-induced rodent models of breast, prostate, lung, esophageal, and skin cancer (Blecha *et al.*, 2007; Lu *et al.*, 2010). NDGA is undergoing early clinical trials for prostate

Table 6.1 Effects of plant (poly)phenols on DNA methyltransferases activity *in vitro*.

Group/class	Compound	Structure	Dietary source	DNMT inhibitory activity[a]	References
Flavones	Apigenin		Celery, chamomile	• Weak DNMT inhibition *in vitro* 20 μM: 30% 50 μM: 37%	Fang *et al.* (2007)
Flavones	Baicalein		*Scutellaria baicalensis*	• DNMT inhibition *in vitro* 20 μM: 80% 40 μM: 80%	Paluszczak *et al.* (2010)
Flavones	Luteolin		Parsley, celery	• DNMT inhibition *in vitro* 20 μM: 33% 50 μM: 55%	Fang *et al.* (2007)

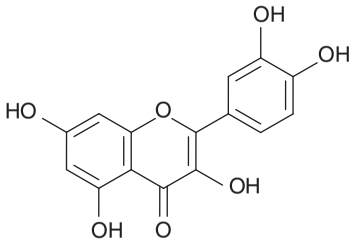
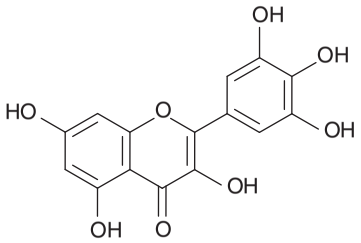
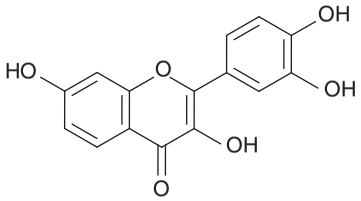
Flavonols	Quercetin		Onion, broccoli, apples, berries	• DNMT inhibition *in vitro* 20 μM: 31% 50 μM: 41% • Possible association with COMT-mediated *O*-methylation leading to SAH formation M.Sss1 (+COMT): IC_{50} 1.6 μM DNMT1 (+COMT): IC_{50} 1.6 μM	Lee *et al.* (2005), Fang *et al.* (2007)
Flavonols	Myricetin		Fruit, herbs, vegetables	• Weak DNMT inhibition *in vitro* 20 μM: 21%, 50 μM: 30% 40 μM: 16% • Possible association with COMT-mediated *O*-methylation leading to SAH formation M.Sss1(+COMT): IC_{50} 1.0 μM DNMT1(+COMT): IC_{50} 3.5 μM	Lee *et al.* (2005), Fang *et al.* (2007), Paluszczak *et al.* (2010)
Flavonols	Fisetin		Strawberries	• DNMT inhibition *in vitro* • Possible association with COMT-mediated *O*-methylation leading to SAH formation M.Sss1: IC_{50} 9.1 μM M.Sss1(+COMT): IC_{50} 0.7 μM DNMT1: IC_{50} 10.7 μM DNMT1(+COMT): IC_{50} 1.2 μM	Lee *et al.* (2005)

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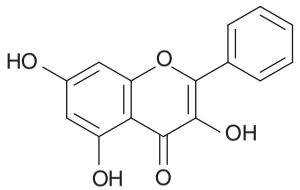
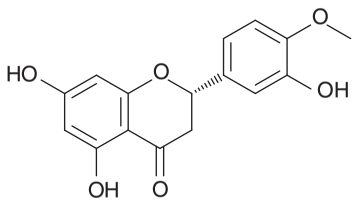
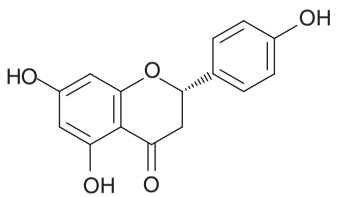
Table 6.1 (*continued*)

Group/class	Compound	Structure	Dietary source	DNMT inhibitory activity[a]	References
Flavonols	Galangin		Propolis, galangal root	• DNMT inhibition *in vitro* 20 μM: 67%	Paluszczak *et al.* (2010)
Flavanones	Hesperetin		Citrus fruit	• DNMT inhibition *in vitro* 20 μM: 26% 50 μM: 49%	Fang *et al.* (2007)
Flavanones	Naringenin		Orange peel	• Weak DNMT inhibition *in vitro* 20 μM: 10% 50 μM: 21%	Fang *et al.* (2007)

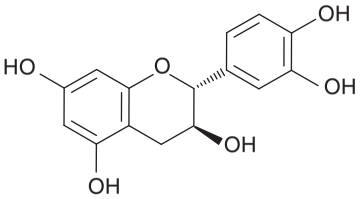
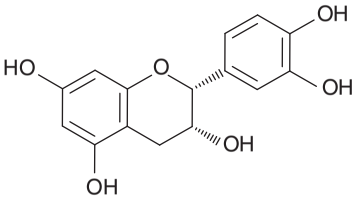
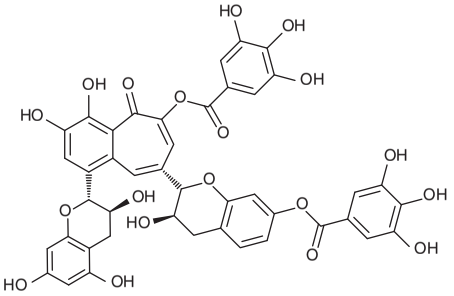
Flavanols (catechins)	Catechin		Green tea	• DNMT inhibition *in vitro* • Possible association with COMT-mediated *O*-methylation leading to SAH formation M.Sss1 (+COMT): IC_{50} 6.5 μM DNMT1 (+COMT): IC_{50} 4.6 μM • ↓ promoter methylation of *RARβ*	Lee *et al.* (2005)
Flavanols (catechins)	Epicatechin		Green tea	• DNMT inhibition *in vitro* • Possible association with COMT-mediated *O*-methylation leading to SAH formation M.Sss1 (+COMT): IC_{50} 8.1 μM DNMT1 (+COMT): IC_{50} 8.4 μM	Lee *et al.* (2005)
Catechin polymer	Theaflavin 3,3′-digallate, thearubigins		Black tea	• DNMT3a inhibtion *in vitro* Theaflavin 3,3′-digallate IC_{50} = 44 μM Thearubigins IC_{50} = 40, 28 μM	Rajavelu *et al.* (2011)

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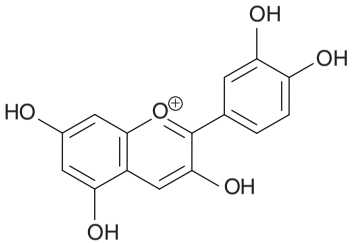
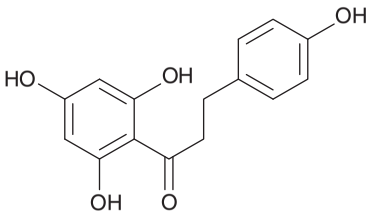
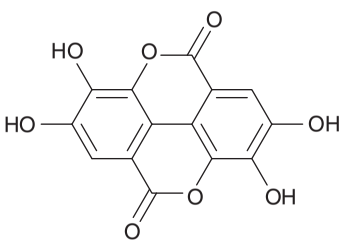
Table 6.1 (*continued*)

Group/class	Compound	Structure	Dietary source	DNMT inhibitory activity[a]	References
Anthocyanidins	Cyanidin		Cherries, strawberries, blueberries, red cabbage, roses	• DNMT inhibition *in vitro* 20 μM: 94% 40 μM: 86%	Paluszczak *et al.* (2010)
Chalcones	Phloretin (dihydrochalcone)		Apples	• DNMT inhibition *in vitro* 20 μM: 81% 40 μM: 27%	Paluszczak *et al.* (2010)
Non-flavonoid polyhenols	Ellagic acid		Berries	• DNMT inhibition *in vitro* 20 μM: 53% 40 μM: 88%	Paluszczak *et al.* (2010)

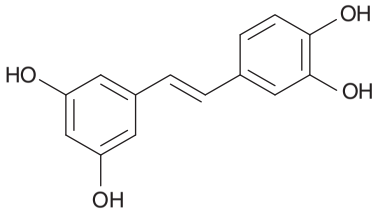
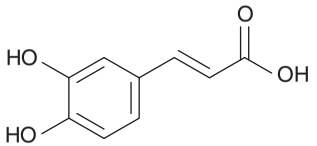
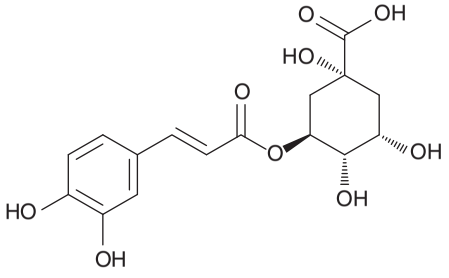
Non-flavonoid polyhenols (Stilbenes)	Piceatannol		Grapes	• Weak DNMT inhibition *in vitro* 20 μM: 32% 40 μM: 31%	Paluszczak *et al.* (2010)
Phenolic acids	Caffeic acid		Coffee	• Non-competitive DNMT1 inhibition *in vitro*, possible association with COMT-mediated *O*-methylation leading to SAH formation M.Sss1 (+COMT): IC_{50} 3.0 μM DNMT1 (+COMT): IC_{50} 2.3 μM • No effect on DNMT3a at 100 μM • Inhibition of HDM[b] KDM4C: IC_{50} 13.7 μM	Lee & Zhu (2006), Rajavelu *et al.* (2011), Nielsen *et al.* (2012)
Phenolic acids	Chlorogenic acid		Coffee	• Non-competitive DNMT1 inhibition *in vitro*, possible association with COMT-mediated *O*-methylation leading to SAH formation M.Sss1 (+COMT): IC_{50} 0.75 μM DNMT1 (+COMT): IC_{50} 0.9 μM	Lee & Zhu (2006)

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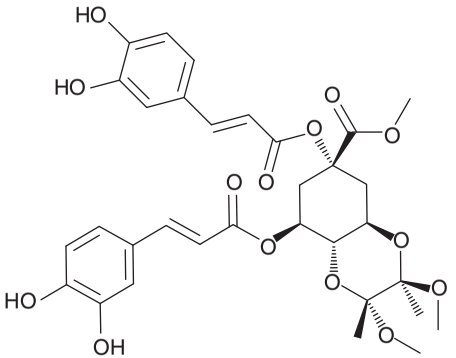
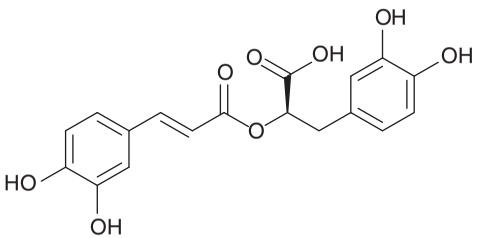
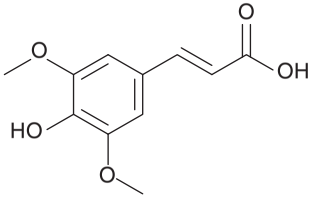
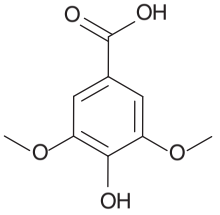
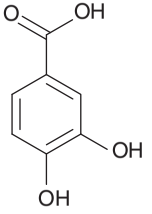
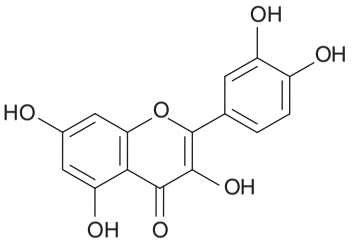
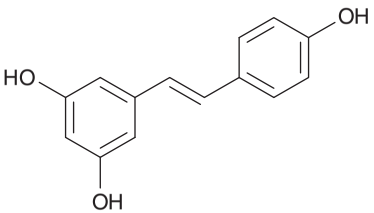
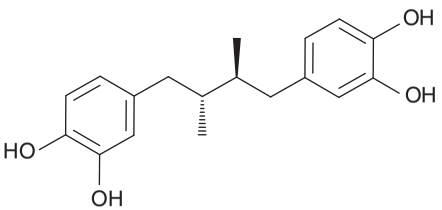
Table 6.1 (*continued*)

Group/class	Compound	Structure	Dietary source	DNMT inhibitory activity[a]	References
Phenolic acids	1,3-dicaffeoyl-muco-quinic acid diacetal		Synthetic A series of 12 chlorogenic acid derivatives screened	• DNMT3a inhibition *in vitro* IC_{50} = 35 μM Only diacyl quinic acids active	Rajavelu *et al.* (2011)
Phenolic acids	Rosmarinic acid		Rosemary	• DNMT inhibition *in vitro* 20 μM: 74% 40 μM: 90%	Paluszczak *et al.* (2010)
Phenolic acids	Hydroxycinnamic acid		Fruit	• DNMT inhibition *in vitro* 20 μM: 37% 50 μM: 74%	Fang *et al.* (2007)

Phenolic acids	Sinapic acid		Rape seed	• DNMT inhibition *in vitro* 20 µM: 58% 40 µM: 61%	Paluszczak *et al.* (2010)
Phenolic acids	Syringic acid		Acai oil, wine	• DNMT inhibition *in vitro* 20 µM: 81% 40 µM: 51%	Paluszczak *et al.* (2010)
Phenolic acids	Protocatechuic acid		Acai oil, olives	• DNMT inhibition *in vitro* 20 µM: 75% 40 µM: 64% • HDM[b] KMD6A inhibition *in vitro*: IC_{50}: 12.0 µM	Paluszczak *et al.* (2010), Nielsen *et al.* (2012)

[a]Fang *et al.* (2007): Reaction based on ^{3}H-labeled SAM as a substrate and poly(dI-dC)·poly(dI-dC) as a template. Paluszczak *et al.* (2010): Activity of DNMTs in nuclear extracts assessed based on the methylation of CG-rich oligonucleotides immobilized in wells; methylated cytosines detected by an ELISA assay. Lee *et al.* (2005): Reaction based on ^{3}H-labeled SAM as a substrate and poly(dI-dC)·poly(dI-dC) as a template. Rajavelu *et al.* (2011): Inhibition of human Dnmt3a catalytic domain analyzed by Biotin–Avidin methylation kinetics assay (Roth & Jeltsch, 2000) using a biotinylated oligonucleotide substrate and ^{3}H-labeled SAM.
[b]HDM, histone lysine demethylase.

Table 6.2 Effects of plant polyphenols on DNA methyltransferases in cell culture and *in vivo*.

Group/class	Compound	Structure	Dietary source	Epigenetic mechanism	References
Flavonols	Quercetin		Onion, broccoli, apples, berries	• ↓ promoter methylation of p16, leading to mRNA and protein re-expression	Tan *et al.* (2009)
Lignans	Nordihydroguaiaretic acid (NDGA)		Creosote bush	• ↓ promoter methylation of p16INK4, leading to mRNA and protein re-expression • ↓ promoter methylation of E-cadherin, leading to mRNA re-expression *in vitro* and *in vivo* • ↔ global (Line1) methylation	Byun *et al.* (2008), Cui *et al.* (2008a,b)
Other polyphenolic compounds (Stilbenes)	Resveratrol		Grapes	• Weak DNMT inhibition *in vitro*[a] 20 μM: 19%, 40 μM: 14% • ↓ MBD2 and DNMT1 recruitment to BRCA1 promoter • ↓ promoter methylation of RARβ, PTEN • ↓ DNMT expression	Paluszczak *et al.* (2010), Papoutsis *et al.* (2010), Stefanska *et al.* (2010, 2012)

Mixed polyphenols	Apple polyphenols	*Annurca* apples	• ↓ DNMT expression • ↓ promoter hypermethylation of hMLH1, p14(ARF), and p16(INK4a) • ↓ global DNA hypomethylation in normal mouse colon and polyps • ↓ promoter hypomethylation of Igf2 and P2rx7 in normal mouse colon	Fini *et al.* (2007, 2011)
Mixed polyphenols	Black raspberry powder	Black raspberries	• ↓ DNMT expression • ↓ promoter hypermethylation of SFRP2, SFRP5, WIF1, and PAX6a in human colon (cancer) biopsies	Wang *et al.* (2011b)

[a]Paluszczak *et al.* (2010): Activity of DNMTs in nuclear extracts assessed based on the methylation of CG-rich oligonucleotides immobilized in wells; methylated cytosines detected by ELISA assay.

cancer treatment. However, hepatotoxicity might limit its use in humans (Ryan *et al.*, 2008; Lu *et al.*, 2010).

NDGA was first reported to reduce global methylation in malignant glioma cells (cited in Cui *et al.*, 2008b). Subsequently, NDGA treatment of breast and colon cancer cell lines resulted in p16 promoter demethylation, and p16 reactivation was associated with cell-cycle arrest and increased senescence (Cui *et al.*, 2008b). Similarly, NDGA reduced promoter hypermethylation and reactivated mRNA expression of the epigenetically silenced epithelial cell marker E-cadherin in breast and colon cancer cell lines, induced re-expression of E-cadherin protein in MDA-MB-435 breast-cancer xenografts, and reduced tumor growth (Cui *et al.*, 2008a,b). In contrast, NDGA treatment in HepG2 liver-cancer cells had no effect on global DNA methylation measured via methylation levels of the repetitive element LINE-1 (Byun *et al.*, 2008).

6.2.3.3 Resveratrol

Resveratrol is a plant-derived stilbene derivative found in the skin of red grapes and in other fruits. It was first identified as a chemopreventive agent in 1997 (Pezzuto, 2008). It has a broad spectrum of health-beneficial effects, including antioxidant, cardioprotective, and antitumor activities, which have mechanistically been linked to effects on hormone receptors, drug metabolism, and cell signaling related to inflammation, cell survival and apoptosis, tumor angiogenesis, and metastasis formation (Kundu & Surh, 2008; Pezzuto, 2008). Resveratrol was shown to prevent carcinogenesis in animal models for breast, skin, esophageal, and colon cancer, and reduced xenograft growth of various tumor cell lines. Although plasma levels after oral consumption are extremely low (Baur & Sinclair, 2006), resveratrol is currently undergoing clinical testing in several small intervention trials (Pezzuto, 2008).

Resveratrol only weakly inhibited DNMT enzymatic activity *in vitro* (Paluszczak *et al.*, 2010). Three recent studies investigated its effect in breast-cancer cell lines. Activation of the aryl hydrocarbon receptor (AhR) has been shown to lead to epigenetic silencing of the DNA repair gene BRCA1 in breast cancer (Papoutsis *et al.*, 2010). This is associated with enhanced binding of repressive histone marks and methyl binding-domain protein 2 (MBD2) to the BRCA1 promoter. MBD2 is known to associate with DNMT1. Papoutsis *et al.* (2010) could demonstrate that pre- or cotreatment with resveratrol reduced MBD2 and DNMT1 levels at the BRCA1 promoter and prevented DNA damage induced by the AhR ligand 2,3,7,8 tetrachlorobenzo(p)dioxin (TCDD).

Retinoid acid receptor (RAR) β2 and the tumor-suppressor PTEN (phosphatase and tensin homolog) are hypermethylated in breast and various other tumor types. Stefanska *et al.* (2010, 2012) used methylation-sensitive restriction analysis to test whether RARβ2 and PTEN silencing could be reversed in breast-cancer cell lines after incubation with resveratrol alone and in combination with nucleoside analogs. Promoters of both genes were about 30% methylated in MCF-7 cells. Incubation with resveratrol reduced methylation levels of both genes; this was associated with down-regulation of DNMT1. However, only PTEN was re-expressed by resveratrol treatment (Stefanska *et al.*, 2010, 2012). This might

indicate that alternative indirect mechanisms other than demethylation are involved in the observed re-expression of PTEN.

6.2.3.4 Apple polyphenols

Apples (*Malus* sp., Rosaceae) are a rich source of polyphenols, including hydroxycinnamic acids, dihydrochalcones, flavonols (quercetin-glycosides), catechins, and oligomeric procyanidins, as well as anthocyanins in red apples and triterpenoids in apple peel (Gerhauser, 2008). Apple polyphenol extracts and isolated components have been shown to possess antimutagenic, antioxidant, and anti-inflammatory activities, to modulate carcinogen metabolism and signal transduction pathways, and to induce apoptosis. In animal models, apple components prevented skin, mammary, and colon carcinogenesis. Epidemiological observations indicate that regular consumption of one or more apples a day may reduce the risk for lung and colon cancer (Gerhauser, 2008).

Fini *et al*. (2007, 2011) asked whether some of the chemopreventive potential of apples might be related to epigenetic mechanisms. They could demonstrate that treatment of colon-cancer cell lines with an *Annurca* apple extract reduced protein expression of DNMT1 and DNMT3b. This was associated with reversal of promoter methylation of the cell-cycle regulators p16(INK4a) and p14(ARF) and the DNA repair gene hMLH1 (human mutL homolog 1), associated with reactivation of mRNA or protein expression (Fini *et al.*, 2007). In the $APC^{Min/+}$ mouse model for intestinal cancer (APC for adenomatous polyposis coli, a master regulator of the so-called Wnt signaling pathway, which is often deregulated in colon cancer; Min for multiple intestinal neoplasia, characterized by the occurrence of large numbers of adenoma in the small intestine), application of an apple extract in drinking water reduced the number of small-intestinal polyps by about 40% and prevented global DNA hypomethylation. Also, age-related hypomethylation of the maternally imprinted mitogenic growth factor insulin-like growth factor 2 (Igf2) and of P2rx7 (P2X purinoceptor 7) was prevented in normal colon by the apple extract, whereas age-related hypermethylation of several genes, including Esr1 (estrogen receptor alpha ERα), Nkx2−5 (NK2 homeobox 5, a homeobox containing cardiac transcription factor), Gpr37 (G protein-coupled receptor 37, also known as endothelin receptor type B-like), Prdm5 (PR domain containing 5, a transcription factor of the PR-domain protein family, known to be involved in cell differentiation and tumorigenesis), Myod1 (myogenic differentiation 1, a basic helix−loop−helix transcription actor), Nptx2 (neuronal pentraxin II), and Igf2-DMR2 (Igf2 differentially methylated region 2), was not affected (Fini *et al.*, 2011).

6.2.3.5 Black raspberry polyphenols

Black raspberries are rich in ellagic acid, quercetin glycosides, and anthocyanins. For more than 20 years, Stoner *et al.* (2007) investigated the *in vitro* and *in vivo* cancer-preventive potential of freeze-dried black raspberries, mainly for esophageal and colon cancer. They could demonstrate that black raspberries influenced carcinogen metabolism, inhibited cell

growth, inflammation, and angiogenesis, and induced apoptosis. In human intervention studies, black raspberries were well tolerated at a dose of 45 g per day for 7 day (Stoner *et al.*, 2007).

In a recent small human phase I pilot study, Wang *et al.* (2011b) provided the first evidence for the modulation of epigenetic markers and downstream effects in human target tissue after chemopreventive intervention. They investigated whether black raspberry intervention (60 g/day for 1–9 weeks) could change the methylation levels of several genes involved in the Wnt signaling pathway, which as already mentioned is often deregulated in colon cancer, including WIF1 (Wnt inhibitory factor 1), sFRP2 and sFRP4 (secreted frizzled-related protein 2 and 4, antagonists of Wnt signaling), the cell-cycle regulator p16, and PAX1 (paired box protein 1, a tissue-specific developmental transcription factor), in normal and cancer tissue of colorectal-cancer patients. After a minimum 4-week intervention, promoter methylation levels of sFRP2 and Pax6 (paired box protein 6, also known as oculorhombin, a transcription factor involved in eye development) were significantly reduced in normal and tumor tissue. Black raspberry uptake also reduced WIF1 promoter methylation in tumor samples, especially after prolonged uptake. These findings were associated with reduced expression of DNMT1 and of downstream Wnt target genes such as β-catenin (a transcription factor activated by aberrant Wnt signaling, often over-expressed in cancer tissue), E-cadherin (a calcium-dependent adhesion protein with high expression in epithelial cells, regulating the cellular distribution of β-catenin), and Ki67 as proliferation markers in tumor tissue (Wang *et al.*, 2011b).

In summary, multiple classes of flavonoids, non-flavonoid polyphenols, and phenolic acids have been screened *in vitro* for DNMT inhibitory activity, using nuclear extracts or recombinant human or bacterial proteins as an enzyme source. In general, inhibitory activities are found at an intermediate μM range. In comparison to the more specific inhibition of other enzyme classes of importance for carcinogenesis (e.g. the inhibition of cytochrome P450 1A1 by quercetin at nanomolar concentrations), it is currently unclear whether the observed inhibitory potential has relevance for changes in gene expression and cancer-preventive activity. In cell culture, reduced expression of DNMT proteins after polyphenol treatment might contribute to inhibitory effects on DNA methylation, independent of direct inhibition of DNMT enzyme activity. With few exceptions, the effects of polyphenols on DNA methylation in animal models and in humans are still limited, and a conclusive link between modulation of DNA methylation levels and cancer-preventive efficacy is often still missing.

6.3 Influence of polyphenols on histone-modifying enzymes

In addition to DNA methylation, chromatin accessibility and gene expression are controlled by various post-translational modifications of N-terminal histone tails, including acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation, and ADP ribosylation (Kouzarides, 2007; Bannister & Kouzarides, 2011; Fullgrabe *et al.*, 2011). Histone acetylation is controlled by the interplay of histone acetyltransferases (HATs) and histone

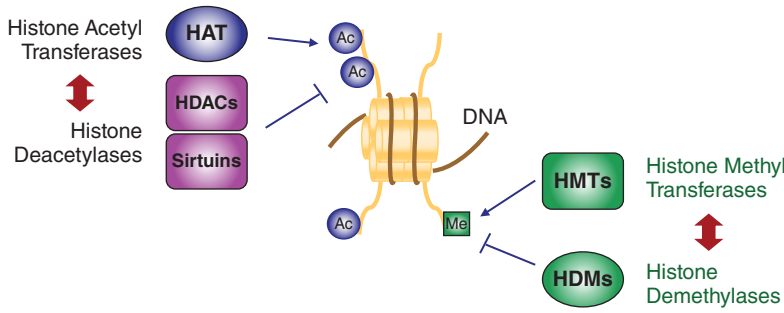


Fig. 6.3 Simplified overview of histone-modifying enzymes, with a focus on histone deacetylases (HDACs), histone acetyltransferases (HATs), histone methyl transferases (HTMs), and histone demethylases (HDMs) and their influence on chromatin structure. Sirtuins represent a NAD^+-dependent subclass of HDACs (class III).

deacetylases (HDACs), whereas histone lysine methylation is controlled by the balance between histone methyltransferases (HMTs) and histone demethylases (HDMs) (Fig. 6.3).

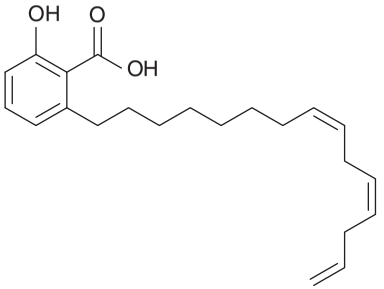
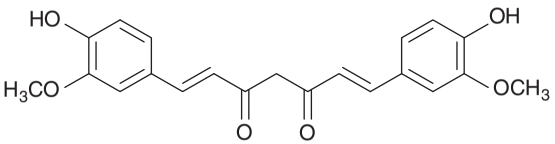
6.3.1 Acetylation of histones and non-histone proteins

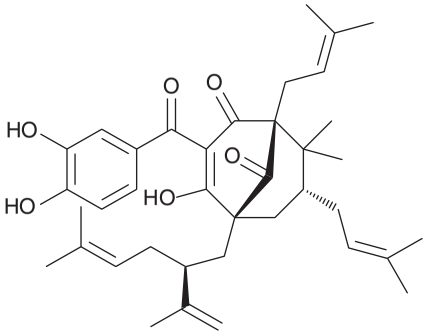
Histone acetylation is catalyzed by HATs, which add acetyl groups from acetyl-coenyzme A as a cofactor to the ϵ-amino group of lysine residues in histone tails (whereas HDACs remove acetyl groups). Histone acetylation leads to an open chromatin structure, allowing transcription factors to access DNA. Consequently, HAT proteins are often transcriptional coactivators, such as p300/CBP (Creb-binding protein) or PCAF (p300/CBP-associated factor). So far, at least 25 proteins with HAT catalytic activity and distinct histone specificity have been identified, subdivided into five subgroups – the GNAT, MYST, p300/CBP, SRC, and TAFII250 families – with varying kinetics of lysine acetylation (Suzuki & Miyata, 2006; Fu & Kurzrock, 2010; Bannister & Kouzarides, 2011; Salvador & Luesch, 2012). Since 2004, several polyphenols have been described as inhibitors of HAT activity, including anacardic acid, curcumin, garcinol, gallic acid, and delphinidin (Table 6.3). The HAT-modulating activities of EGCG and genistein will be described in Section 6.5.

6.3.1.1 Anacardic acid

Anacardic acid (6-nonadecyl salicylic acid) is a component of cashew nut shell liquid (Hemshekhar *et al.*, 2012) and was identified in an *in vitro* inhibition screen as the first natural-product inhibitor of p300 and PCAF HAT activity, with IC_{50} values of 8.5 and 5.0 µM respectively (Balasubramanyam *et al.*, 2003). Anacardic acid also inhibits the HAT enzyme Tip60 (a member of the MYST family), which is involved in the activation of key enzymes of DNA damage response, with an IC_{50} value of 9 µM (Sun *et al.*, 2006). Recent simulations of molecular docking to the p300 active site indicate that anacardic acid mimics binding of the cofactor acetyl-CoA, suggesting that it nonspecifically inhibits HAT activity (Ghizzoni *et al.*, 2010). Interestingly, anacardic acid also inhibited p300-mediated

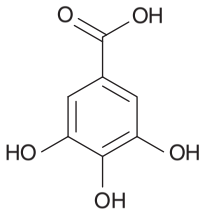
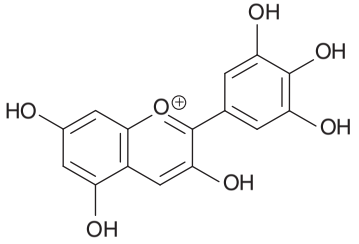
Table 6.3 Effects of plant (poly)phenols on histone acetyl transferases (HAT).

Group/class	Compound	Structure	Dietary source	Epigenetic mechanism	References
Phenolic acids	Anacardic acid		Cashew nuts	• HAT inhibition *in vitro* IC_{50} p300: 8.5 μM IC_{50} PCAF: 5 μM IC_{50} Tip60: 9 μM • ↓ acetylation and nuclear translocation of NF-κB p65	Balasubramanyam *et al.* (2003), Sun *et al.* (2006), Sung *et al.* (2008)
Phenolic acid derivative	Curcumin (diferuloyl-methane)		Turmeric (*Curcuma longa*)	• HAT inhibition *in vitro* IC_{50} p300: ~19.5 μM IC_{50} CBP: ~22.5 μM • ↓ histone and p53 acetylation No effect on PCAF, HDAC, HMT activity *in vitro*	Balasubramanyam *et al.* (2004b), Kang *et al.* (2005)
				↓ HAT p300 expression • ↓ NF-κB p65 acetylation	Chen *et al.* (2007), Ryu *et al.* (2008), Yun *et al.* (2011)
				↓ HDAC1,2,3,4,8 expression • ↑ ac-H4 • ↑ tubulin acetylation • ↓ NF-κB activity	Liu *et al.* (2005), Chen *et al.* (2007), Lee *et al.* (2011), Yun *et al.* (2011)

				↓ HMT EZH2 expression • ↓ H3K27me3	Hua *et al.* (2010), Shu *et al.* (2011)
				↓ DNMT activity, expression	Liu *et al.* (2009, 2011a,b), Kuck *et al.* (2010), Abusnina *et al.* (2011), Khor *et al.* (2011), Medina-Franco *et al.* (2011), Shu *et al.* (2011)
				↓ promoter methylation of several genes in cell culture	Jha *et al.* (2010), Khor *et al.* (2011), Liu *et al.* (2011a,b), Shu *et al.* (2011), Vilas-Zornoza *et al.* (2011)
Non-flavonoid polyphenols	Garcinol		Mangosteen tree *Garcinia indica*	• HAT (p300, PCAF) inhibition *in vitro* IC_{50} p300: 7 μM IC_{50} PCAF: 5 μM • ↓ H3 and H4 acetylation • ↓ global gene expression in HeLa cells • DNMT inhibition *in vitro*[a] 20 μM: 36% 50 μM: 65%	Balasubramanyam *et al.* (2004a), Fang *et al.* (2007), Arif *et al.* (2009)

(*continued overleaf*)

Table 6.3 (*continued*)

Group/class	Compound	Structure	Dietary source	Epigenetic mechanism	References
Phenolic acids	Gallic acid		Fruit, berries, tea, coffee, oak bark, walnuts, witch hazel, sumach	• HAT (p300, CBP, PCAF, Tip60) inhibition *in vitro* IC_{50} p300: 14 μM IC_{50} CBP: 24 μM IC_{50} PCAF: 34 μM IC_{50} Tip60: 25 μM • No effect on SIRT1, HDAC, and HMT activities • No effect on DNMT3a at 100 μM	Choi *et al.* (2009b), Rajavelu *et al.* (2011)
Anthocyanidins	Delphinidin		Dark fruit and berries, e.g. blueberries	• Specific HAT (*p300*) inhibition *in vitro* IC_{50} *p300*: ~30 μM • ↔ no effect on PCAF, SIRT1, HDAC, HMT activities • ↓ acetylation of NF-κB p65	Seong *et al.* (2011)

[a] Fang *et al.* (2007): Reaction based on 3H-labeled SAM as a substrate and poly(dI-dC)·poly(dI-dC) as a template.

acetylation of the p65 subunit of NF-κB (nuclear factor "kappa light-chain enhancer" of activated B cells) as a non-histone substrate of HATs, and inhibited NF-κB-mediated signaling involved in cell survival, proliferation, invasion, and inflammation (Sung *et al.*, 2008). A disadvantage of anacardic acid might be its low cellular permeability (Balasubramanyam *et al.*, 2003).

6.3.1.2 Curcumin

The yellow pigment curcumin (diferuloyl methane) is a component of turmeric (*Curcuma longa*) and a major ingredient of the spice curry. Curcumin belongs to the most actively investigated cancer chemopreventive agents and has a long-term use in traditional medicine in India and other countries (Gupta *et al.*, 2013b). It has a broad spectrum of activities and acts on multiple signaling pathways, particularly NF-κB signaling. Curcumin has been shown to induce apoptosis and block invasion, metastasis, and angiogenesis *in vitro* and to prevent or inhibit tumor growth in rodent models, essentially for all major tumor entities (Surh & Chun, 2007; Kunnumakkara *et al.*, 2008; Ravindran *et al.*, 2009; Gupta *et al.*, 2013a,b). Investigations of curcumin in healthy human subjects and in patients with inflammatory diseases and cancer have shown promising first results and demonstrate that the compound is well tolerated even at elevated concentrations of up to 15 g/day (Kunnumakkara *et al.*, 2008; Gupta *et al.*, 2013a,b).

Curcumin was first identified as an inhibitor of HAT activity in 2004. Balasubramanyam *et al*. (2004b) described a decrease in histone H4 acetylation in Raji cell culture after curcumin treatment and identified curcumin as a specific inhibitor of p300/CBP, whereas PCAF, HDAC, and HMT activities were not affected by curcumin even at elevated concentrations. Curcumin also inhibited acetylation of the tumor suppressor protein p53 as a non-histone protein target of p300/CBP (Balasubramanyam *et al.*, 2004b). Acetylation of p53 was shown to be essential for p53-dependent growth arrest and apoptosis (Tang *et al.*, 2008b). HAT p300 inhibition was confirmed by Kang *et al*. (2005), and exposure of human hepatoma cells to curcumin led to a significant decrease in histone acetylation, as would be expected from HAT inhibition. This histone hypoacetylation was suggested to involve increased production of reactive oxygen species (ROS) by curcumin treatment and was blocked by antioxidants.

In addition to direct inhibition of HAT activity, treatment with curcumin and related compounds reduced the expression of p300. This was associated with downstream effects on Wnt- and NF-κB signaling (Chen *et al.*, 2007; Ryu *et al.*, 2008; Yun *et al.*, 2011). Curcumin treatment also led to a reduced expression of HDACs 1, 2, 3, 4, and 8, and consequently reduced cell proliferation *in vitro* and *in vivo* (Liu *et al.*, 2005; Chen *et al.*, 2007; Lee *et al.*, 2011; Yun *et al.*, 2011). Recent studies point to an effect of curcumin on HMT activities. Expression of EZH2 (enhancer of zeste homolog 2), a member of the polycomb repressive complex 2 (PRC2), and levels of the repressive histone mark H3K27me3 were reduced in breast- and prostate-cancer cell lines after curcumin treatment (Hua *et al.*, 2010; Shu *et al.*, 2011).

Liu *et al.* (2009) performed *in silico* docking studies to the homology domain of DNMT and identified curcumin and related analogs as putative DNMT inhibitors and DNA demethylating agents; this was later confirmed by Medina-Franco *et al.* (2011).

Subsequently, curcumin and its analogs were shown to inhibit DNMT activity *in vitro* (Kuck *et al.*, 2010; Liu *et al.*, 2011a,b) or to reduce DNMT expression (Abusnina *et al.*, 2011). Sequence-specific demethylation at the promoter regions of epigenetically silenced genes was demonstrated in various cancer cell lines (Jha *et al.*, 2010; Khor *et al.*, 2011; Liu *et al.*, 2011a,b; Shu *et al.*, 2011; Vilas-Zornoza *et al.*, 2011).

A recent study by Link *et al.* (2010) performed genome-wide profiling of DNA methylation changes in three colon-cancer cell lines after short- (6 days) and long-term (240 days) exposure to curcumin, using the Illumina 27 k bead array technology. These arrays covered roughly 27 000 CpG sites associated with 14 000 genes. Long-term incubation with curcumin at concentrations leading to about 50% reduction of cell growth led to changes in DNA methylation of 814–3051 individual CpG sites, with about equal distribution of hyper- and hypomethylation events. Sixty-eight loci were hypomethylated in all three cell lines. In contrast to the global demethylation observed with the demethylating agent 5-aza-2′-deoxy-cytidine (decitabine), curcumin mainly targeted CpG sites with intermediate methylation levels. Comparison of methylation with gene-expression changes in long-term treated cell lines indicated that genes involved in cell metabolism, cell signaling, cell proliferation, cell-, tissue-, and cancer development, amongst others, were regulated by curcumin via changes in DNA methylation. Mechanistically, the authors interpreted long-term curcumin effects on DNA methylation as a DNMT-independent event. Instead, they suggested that long-term silencing of important transcription factor-mediated pathways might allow passive gain of methylation at CpG sites that no longer interacted with transcription factors. Overall, short-term curcumin treatment had only negligible effects.

Curcumin and its derivatives have also been identified as potent modulators of miRNAs (Li *et al.*, 2010a; Izzotti *et al.*, 2012; Gupta *et al.*, 2013a). Further details on gene regulation and the activities of curcumin through epigenetic mechanisms have been summarized in several comprehensive review articles (Fu & Kurzrock, 2010; Li *et al.*, 2010a; Huang *et al.*, 2011; Reuter *et al.*, 2011; Khan *et al.*, 2012; Vanden Berghe, 2012).

6.3.1.3 Garcinol

The polyisoprenylated benzophenone garcinol was isolated from the mangosteen tree *Garcinia indica* Choisy (*Clusiaceae*). A chemopreventive effect was attributed to its antioxidant and anti-inflammatory activities. It induced phase II detoxifying enzymes and inhibited cell proliferation through induction of apoptosis. *In vivo*, it prevented colon and tongue cancer (Padhye *et al.*, 2009).

Garcinol was identified as a cell-permeable HAT inhibitor active against PCAF and p300 with IC_{50} values of 5 and 7 μM, respectively. It was shown to act as a noncompetitive and competitive inhibitor of acetyl-CoA and the histone substrate (Salvador & Luesch, 2012). Molecular-docking studies suggested that the phenol moiety facilitates binding to the acetyl-CoA pocket, whereas the prenyl group substitutions affect specificity and binding to allosteric HAT sites (Arif *et al.*, 2009; Salvador & Luesch, 2012). In HeLa cells, garcinol repressed histone acetylation and global gene expression and induced apoptosis (Balasubramanyam *et al.*, 2004a). A recent study suggests that garcinol also

affects miRNAs of the miR-200 and let-7 families and may reduce the metastatic potential of aggressive breast-cancer cells through reversion of epithelial–mesenchymal transition (EMT) (Ahmad *et al.*, 2012).

6.3.1.4 Gallic acid

Gallic acid (3,4,5-trihydroxybenzoic acid) is found in various fruit and berries, tea and coffee, oak bark, walnuts, witch hazel (*Hamamelis virginiana*), sumach (*Rhus coriaria*), and other plants, both free and as part of hydrolyzable tannins (gallotannins) (Choi *et al.*, 2009b; Ferk *et al.*, 2011). Gallic acid has good antioxidant activity both *in vitro* and *in vivo*, partly through increasing the activity of antioxidant enzymes (Ferk *et al.*, 2011). It has been shown to reduce oxidative DNA damage (Ferk *et al.*, 2011) and to induce apoptosis preferentially in cancer cells by various mechanisms (Locatelli *et al.*, 2012).

In a recent study by Choi *et al*. (2009b), gallic acid was identified as a specific inhibitor of HAT activity *in vitro*, without affecting other epigenetic enzymes. IC_{50} values for p300, CBP, PCAF, and Tip60 were in the range 14–34 μM. Like anacardic acid, gallic acid inhibited the p300-mediated acetylation of RelA (v-Rel reticuloendotheliosis viral oncogene homolog A), the p65 subunit of NF-κB, and subsequently reduced NF-κB activation and expression of anti-apoptotic genes in response to pro-inflammatory stimuli (Choi *et al.*, 2009b).

6.3.1.5 Delphinidin

Delphinidin is an anthocyanidin that occurs at high concentrations (about 10× higher than other flavonoids such as quercetin) in dark fruit, particularly blueberries. Anthocyanidins have high antioxidant potential and induce detoxifying enzymes. They often possess antiproliferative activity and induce apoptosis and cell differentiation. Inhibition of angiogenesis and invasiveness also contributes to their chemopreventive potential (Wang & Stoner, 2008). Anthocyanidins have been shown to prevent cancer in rodent models for esophageal, lung, skin, and colon cancer. In human studies, intervention with anthocyanidin-rich fruit powders or fruit juices reduced levels of oxidative stress markers, but results on cancer-preventive potential from epidemiological studies remain inconclusive. This might be associated with low absorption and rapid elimination of anthocyanidins or their naturally occurring glycosides (Wang & Stoner, 2008).

Seong *et al*. (2011) identified delphinidin as a HAT inhibitor. The IC_{50} value for inhibition of p300 activity was determined as 30 μM. Delphinidin also inhibited CBP activity, whereas PCAF and other epigenetic enzymes were not affected. Like gallic acid, delphinidin reduced pro-inflammatory signaling by preventing acetylation of the NF-κB subunit p65.

Overall, HAT-mediated acetylation of histones and non-histone proteins seems to play an important role in regulating inflammatory signaling, in particular the NF-κB pathway, and inhibition of HAT activity might contribute to the anti-inflammatory activity of chemopreventive polyphenols (Ghizzoni *et al.*, 2011).

6.3.2 Deacetylation by HDACs and sirtuins

HDACs catalyze the transfer of acetyl groups from acetylated histones or other acetylated proteins to coenzyme A. In general, histone deacetylation leads to chromatin condensation and transcriptional repression. Eighteen proteins with HDAC activity have been classified so far. HDACs 1–11 are subdivided into three classes – I, II, and IV – based on homology, size, subcellular expression, and number of enzymatic domains. Class III comprises sirtuins 1–7, which are structurally unrelated to class I and II HDACs and require NAD^+ as a cofactor for activity (Sauve *et al.*, 2006; Mottet & Castronovo, 2008). In addition to histones, important regulatory non-histone proteins and transcription factors such as p53, E2F, NF-κB, FOXO (forkhead-box-protein O3), and PGC-1α (peroxisome proliferator-activated receptor (PPAR) gamma, coactivator 1 alpha) involved in inflammation, stress response, apoptosis, and energy metabolism have been shown to be regulated by acetylation (Kim *et al.*, 2006; Sauve *et al.*, 2006; Mottet & Castronovo, 2008; Ghizzoni *et al.*, 2011; Li & Kazgan, 2011).

6.3.2.1 Inhibition of HDAC activity

Structure–activity-relationship analyses and molecular-docking studies have defined structural requirements for HDAC inhibition. The active center of an HDAC is characterized by the presence of a Zn^{2+} ion and lies within a pocket that accommodates the lysine residue to be acetylated. Consequently, HDAC inhibitors generally possess (i) a metal-binding functional group that interacts with the Zn^{2+} ion at the active site, (ii) a hydrocarbon linker that mimics the lysine residue, and (iii) a cap group that interacts with amino acids at the entrance of the active-site pocket (Bora-Tatar *et al.*, 2009) to stabilize the interaction. Spatial constraints might explain why, so far, complex multiring structured flavonoid-type polyphenols have not been identified as inhibitors of HDAC enzymatic activity. However, as described in Section 6.3.1.2 for curcumin, and as outlined in later sections for compounds such as EGCG and genistein, polyphenols have been reported to modulate HDAC activity by affecting the expression of various HDAC isoenzymes.

In a screen of 33 carboxylic-acid derivatives for inhibition of HDAC activity, chlorogenic acid and curcumin were identified as HDAC inhibitors, with IC_{50} values of 375 and 115 μM, respectively. This was equivalent to HDAC inhibition mediated by the short-chain fatty acid (SCFA) butyric acid (IC_{50} 116 μM) and its salt, sodium butyrate (IC_{50} 365 μM), well-known natural HDAC inhibitors. Inhibitory potential was substantiated by molecular docking to the HDAC8 binding cavity (Bora-Tatar *et al.*, 2009). It should be noted that inhibitory concentrations are relatively high and might not be reached *in vivo* after consumption of dietary sources of chlorogenic acid or curcumin.

6.3.2.2 Modulation of sirtuin activity

Silent information regulator 1 (SIRT1, sirtuin 1) has been shown to participate in various cellular processes, from fat and glucose metabolism to aging and cancer (Lavu *et al.*, 2008), but its role in carcinogenesis is still poorly understood (Brooks & Gu, 2009; Stunkel & Campbell, 2011). SIRT1 negatively affects stress response, but is also involved in recovery

from DNA damage. Interestingly, over-expression of SIRT1 is regarded as oncogenic in the presence of wild-type (wt) p53, but is tumor-suppressive in tumors with mutant p53 (Huang *et al.*, 2011).

Interest in SIRT1 was raised when Howitz *et al*. (2003) identified the chemopreventive agent resveratrol (see Section 6.2.3.3) as an activator of SIRT1 and demonstrated that resveratrol increased lifespan in yeast and *C. elegans*, mimicking the effects of calorie restriction (Cohen *et al.*, 2004; for a recent review, see Farghali *et al.*, 2013). Since then, other polyphenols have been identified as activators (silymarin) or inhibitors (3,2′,3′,4′-tetrahydroxy-chalcone) of sirtuins (Table 6.4).

SIRT1 activator: flavolignan silymarin from milk thistle

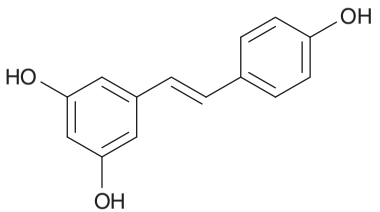
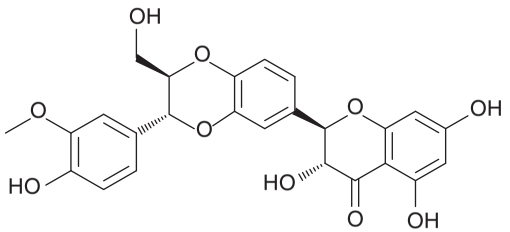
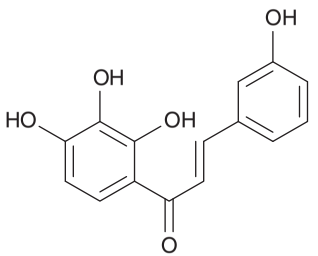
Milk thistle (*Silybum marianum*) is traditionally used to protect against various liver diseases. Silymarin derived from milk-thistle seeds contains at least seven flavolignans and additional compounds. The most abundant component is silybinin (or silibinin), which constitutes a mixture of two isomers, silybin A and B. Although often used synonymously, silymarin and silybinin show distinct activity profiles (Kroll *et al.*, 2007). Cancer-preventive potential *in vitro* and *in vivo* has been attributed to the inhibition of inflammation, cell growth, angiogenesis, tumor invasion, and metastasis (Ramasamy & Agarwal, 2008; Loguercio & Festi, 2011). In clinical trials, milk-thistle extracts were shown to delay progression of prostate cancer (Tamayo & Diamond, 2007).

In a recent study, silymarin was identified as an activator of SIRT1 in human melanoma cells (Li *et al.*, 2007). SIRT1 activation was associated with cell survival and protected the cells from UV light-induced apoptosis. On the other hand, Ciu *et al.* (2009) reported that silybinin treatment reduced the growth of human liver cancer xenografts through induction of apoptosis, and this was associated, besides effects on PTEN/AKT (v-akt murine thymoma viral oncogene homolog 1, also known as protein kinase B, PKB) and ERK (extracellular signal-regulated kinase) signaling, with an increase in histone H3 and H4 acetylation. The underlying mechanism for histone hyperacetylation (e.g. HDAC inhibition versus HAT activation) was not elucidated.

SIRT inhibitor 3,2′,3′,4′-tetrahydroxychalcone

In an attempt to identify novel activators or inhibitors of SIRT1, Kahyo *et al*. (2008) tested a series of polyphenols, including 10 stilbenes, 8 chalcones, 10 flavanones, and 8 flavonols, using purified recombinant SIRT1 enzyme and an acetylated peptide of p53 as a substrate for the deacetylation reaction. At a test concentration of 100 μM, three hydroxy-chalcones inhibited SIRT1 deacetylase activity by more than 90%. Further testing using crude cell lysates indicated that only one of the chalcones, synthetic 3,2′,3′,4′-tetrahydroxychalcone, efficiently inhibited deacetylation of full-length p53. IC_{50} values for SIRT1 and 3 inhibition were calculated as 40.3 and 48.0 μM, respectively. In human embryonic kidney cells, the chalcone dose-dependently induced hyperacetylation of endogenous p53 and increased protein expression of the p53 target p21. This was associated with suppression of cell growth (Kahyo *et al.*, 2008). Since the chalcone was a more effective inhibitor than the known SITR1 inhibitor sirtinol, further testing of its cancer-preventive efficacy is warranted.

Table 6.4 Effects of plant polyphenols on sirtuins.

Group/class	Compound	Structure	Dietary source	Epigenetic mechanism	References
Non-flavonoid polyphenols (Stilbene)	Resveratrol		Grapes	• Activation of SIRT1	Howitz *et al.* (2003)
Flavonolignan	Silymarin		Milk thistle	• SIRT activation • ↑ ac-H3 and ac-H4	Li *et al.* (2007), Cui *et al.* (2009)
Chalcones	Chalcone derivative 3,2′,3′,4′-tetrahydroxy-chalcone		synthetic	• SIRT inhibition *in vitro* • IC_{50} SIRT1: 40.3 µM • IC_{50} SIRT3: 48 µM • IC_{50} HDACs: 445 µM • ↑ p53 acetylation	Kahyo *et al.* (2008)

6.3.3 Histone methylation marks

Besides histone acetylation, histone methylation is an important epigenetic marker involved in the regulation of chromatin structure. Histone methylation takes place at lysine and arginine residues. Histone lysine methylation has activating or repressive effects on gene expression, dependent on the lysine residue that is methylated (e.g. K4, K9, K27, K36, K79 in H3), the methylation status (mono-, di-, or trimethylation), and the location (interaction with promoter versus gene-coding regions) (Kouzarides, 2007; Bannister & Kouzarides, 2011; Brait & Sidransky, 2011). Methylation at H3K4, H3K36, and H3K79 is generally associated with transcriptional active chromatin (*euchromatin*), whereas methylation at H3K9, H3K27, and H4K20 is frequently associated with transcriptional inactive heterochromatin (Suzuki & Miyata, 2006; Upadhyay & Cheng, 2011).

6.3.3.1 Histone lysine methylation

Histone lysine methylation is mediated by histone lysine methyltransferases (HMTs), which transfer a methyl group from SAM to the lysine residue. HMTs can be classified as belonging to the Dot1 (disruptor of telomeric silencing) protein family and as proteins containing a so-called SET domain, based on sequence similarity with the *Drosophila* proteins SUV (suppressor of variegation), EZH (enhancer of zeste), and TRX (homeobox gene regulator trithorax). So far, more than 50 SET-domain family members, which can be grouped in six subfamilies, have been identified in humans (Upadhyay & Cheng, 2011).

Synthetic HMT inhibitors are developed based on structural similarity with the substrate SAM. So far, only a few natural compounds have been described as affecting histone lysine methylation (Huang *et al.*, 2011). The alkaloid chaetocin was one of the first selective inhibitors of the SUV39 class of HMTs targeting H3K9 to be identified (Ellis *et al.*, 2009). Trimethylation of H3K9 is generally associated with repressed chromatin. n-3 polyunsaturated fatty acids (PUFAs) were shown to target EZH. EZH2 is over-expressed in various cancer types, including breast and prostate cancer, and promotes H3K27 trimethylation and thereby maintains the transcriptional repressive states of genes. n-3 PUFAs were shown to reduce the expression of EZH2 by increasing its proteasomal degradation (Dimri *et al.*, 2010). As described in Section 6.5.1.2, the tea polyphenol EGCG also reduces the expression of several HMTs in various cancer cell lines (Balasubramanian *et al.*, 2010).

6.3.3.2 Histone lysine demethylation

Several types of histone lysine demethylase (HDM) have been identified so far, including lysine-specific demethylase 1 (LSD1, also known as KDM1A and B) and a family of about 20 Jumonji domain-containing (JmjC) HDMs (Kouzarides, 2007; Bannister & Kouzarides, 2011; Upadhyay & Cheng, 2011). LSD1 is a FAD (flavin adenine dinucleotide, a coenzyme and electron carrier)-dependent amine oxidase, which removes methyl groups from mono- or dimethylated H3K4 or H3K9 through a FAD-dependent oxidation reaction that leads to the formation of H_2O_2 and formaldehyde. This demethylation alters the chromatin structure and results in context-dependent activation or repression of transcription (Teperino *et al.*, 2010). Like lysine acetylation, lysine methylation is not limited to histone proteins,

and several non-histone protein substrates, including p53, retinoblastoma protein (RB), the NF-κB subunit RelA, and estrogen receptor α (ERα), have been identified (Yang *et al.*, 2009b; Munro *et al.*, 2010; West & Gozani, 2011).

In a screen to detect LSD1 inhibitors, two polyamine analogs, PG11144 and PG11150, were identified as potent competitive inhibitors with IC_{50} values of 5 μM (Huang *et al.*, 2009). In a study by Nielsen *et al.* (2012) to discover novel HDM inhibitors, a natural product library representing 640 compounds was screened against the HDM KDM4C (lysine (K)-specific demethylase 4C, also known as GASC1/JmjD2C), a putative oncogene; a subset was further tested against two additional HDMs, KDM6A (lysine (K)-specific demethylase 6A, involved in cellular differentiation), and PHF8 (PHD finger protein 8, another putative oncogene over-expressed in prostate cancer). A short biotin-labeled histone tail fragment with trimethylation at lysine 9 was used as a substrate, and the resulting H3K9me2 fragment was detected with a specific antibody. Caffeic acid (Table 6.1) was identified as an inhibitor of both KDM4C and KDM6A, with IC_{50} values of 13.7 and 5.5 μM, respectively. Protocatechuic acid (PCA, Table 6.1) was more potent in inhibiting KDM6A than KDM4C, with IC_{50} values of 12.0 and 52.0 μM. None of the compounds inhibited PHF-8 activity, showing that the effects were not a result of general unspecific binding or inhibition. Mechanistic analyses of the inhibitory potential of PCA indicated a mixed mode of inhibition with respect to the peptide substrate, and a noncompetitive mode with respect to the 2-oxoglutarate cofactor. The authors concluded that the compound might interact with the peptide instead of binding to the cofactor pocket. In human osteosarcoma cells over-expressing KDM4C, PCA treatment at concentrations in the range 0.156–2.500 mM indeed inhibited the decrease in H3K9 trimethylation levels, although it should be noted that the concentrations used for testing were extremely high.

6.4 Influence of noncoding miRNAs on gene expression

MiRNAs are small, noncoding RNAs with a length of 20–22 nucleotides that regulate gene expression at the post-transcriptional level by controlling the translation of mRNA into proteins. They either bind to the 3′-untranslated region of mRNA in order to repress protein synthesis or affect mRNA stability. Each miRNA is estimated to control several hundred genes involved in key biological processes, including development, differentiation, apoptosis, and proliferation (Calin & Croce, 2006). MiRNAs are produced from RNA precursor structures by a complex multistep process involving proteins of the Argonaute family, polymerase II-dependent transcription, and the ribonucleases Drosha and Dicer (Winter *et al.*, 2009). Recent research indicates that miRNA expression is altered in various chronic degenerative diseases, including cancer (Calin & Croce, 2006). During carcinogenesis, miRNAs can be aberrantly up-regulated (oncomiRs) or down-regulated (tumor-suppressor miRs). Major mechanisms contributing to their deregulation include genetic and epigenetic alterations such as DNA methylation and defects in the miRNA processing machinery (Brait & Sidransky, 2011).

Several chemopreventive polyphenols have been shown to modulate gene expression via miRNAs. Since a number of recent reviews comprehensively summarize the action of dietary chemopreventive agents on miRNAs (Davis & Ross, 2008; Li *et al.*, 2010a; Link *et al.*, 2010; Saini *et al.*, 2010; Che & Huang, 2012; Izzotti *et al.*, 2012; Karius *et al.*, 2012; Neelakandan *et al.*, 2012; Parasramka *et al.*, 2012; Shah *et al.*, 2012; Lancon *et al.*, 2013; Sethi *et al.*, 2013; Wang *et al.*, 2013; Yi *et al.*, 2013), this section will not cover the activities of polyphenols on miRNAs and miRNA-mediated gene expression. Exceptions are EGCG and genistein, which are described in more detail in the following section.

6.5 Chemopreventive polyphenols affecting the epigenome via multiple mechanisms

Several polyphenols with chemopreventive efficacy *in vitro* and *in vivo* affect the epigenome by multiple and eventually interacting mechanisms. Two compounds will be highlighted in this review: the catechin EGCG from green tea (*Camellia sinensis* L. Kuntze) and the isoflavone genistein from soy (*Glycine max* L. Merr.) (Fig. 6.4).

6.5.1 (−)-epigallocatechin 3-gallate (EGCG) and green-tea polyphenols (GTPs)

Green−tea polyphenols (GTPs) constitute a mixture of flavan-3-ols containing a catechol moiety. The main GTPs are EGCG, epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). EGCG has a broad spectrum of chemopreventive activities *in vitro*, including activity as a pro- and antioxidant, effects on various signal-transduction pathways and receptor-dependent signaling cascades (MAPK, EGFR, NF-κB, IGF), inhibition of enzyme activities involved in drug metabolism and inflammation, and inhibition of cell growth and angiogenesis (Khan & Mukhtar, 2008). *In vivo*, EGCG and GTP prevented carcinogenesis in animal carcinogenesis models of all major organ sites, including lung,

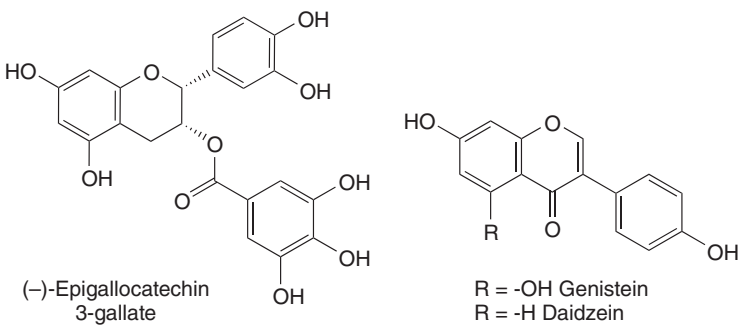


Fig. 6.4 Chemical structures of (−)-epigallocatechin 3-gallate (ECGC), genistein, and daidzein.

colon, breast, prostate, skin, stomach, and liver cancer and tumors of the oral cavity. So far, human epidemiological studies have provided less conclusive results, most likely due to the low quantities of tea consumed (Yang *et al.*, 2009a).

6.5.1.1 DNA methylation

In a landmark study, Fang *et al.* (2003) described the DNA demethylating activity of EGCG (Table 6.5). Using nuclear extracts as an enzyme source, they suggested that EGCG is a competitive inhibitor of DNMT activity. Based on *in silico* modeling using a homology model of the catalytic domain of human DNMT1, EGCG was postulated to directly bind to the catalytic pocket, stabilized by hydrogen bonding with key amino acids. This was supported by two subsequent studies (Lee *et al.*, 2005; Yoo & Medina-Franco, 2011). Lee *et al.* (2005) demonstrated that Mg^{2+} enhances the potential of EGCG to inhibit human DNMT by stabilizing the interaction between Glu1265 and the gallic acid moiety of EGCG. In several studies, DNMT activity was reduced in cultured cells treated with EGCG (Pandey *et al.*, 2009; Meeran *et al.*, 2011, 2012; Nandakumar *et al.*, 2011); this was partly associated with a reduced expression of DNMT mRNA or protein. EGCG or GTP intervention *reduced* genomic 5-methyl cytosine levels in A431 and Jurkat cell lines (Nandakumar *et al.*, 2011; Wong *et al.*, 2011) but was not effective at reducing global methylation levels or methylation of repetitive elements in two other studies (Chuang *et al.*, 2005; Stresemann *et al.*, 2006). On the other hand, GTP *enhanced* methylation of LINE-1 repetitive elements in LNCaP prostate-cancer cells (Pandey *et al.*, 2009). Since loss of global methylation has been associated with enhanced genomic instability, an increase of methylation at repetitive elements might indicate a preventive effect.

Several studies investigated whether EGCG treatment of cultured cells would lead to promoter methylation and subsequent re-expression of selected candidate genes. In KYSE510 esophageal and HT29 colon cancer cells, incubation with EGCG led to promoter demethylation and re-expression of the cell-cycle inhibitor p16 (Fang *et al.*, 2003, 2007). These results could be confirmed in A431 epidermoid carcinoma cells (Nandakumar *et al.*, 2011), but not in T24, HT29, or PC3 cells (Chuang *et al.*, 2005). EGCG treatment resulted in reduced promoter methylation and mRNA/protein re-expression of the DNA repair genes MGMT (*O*-6-methylguanine-DNA methyltransferase) and hMLH1 and of the retinoic acid receptor RARβ2 in human esophageal carcinoma cells (Fang *et al.*, 2003, 2007). In breast-cancer cell lines, RARβ2 promoter methylation was slightly reduced by EGCG intervention (Lee *et al.*, 2005).

Human telomerase reverse transcriptase (hTERT) is a catalytic subunit of the enzyme telomerase and is often upregulated in cancer cells. In normal cells, hTERT transcription is generally repressed through binding of the repressor E2F to its promoter region, whereas in cancer cells binding of E2F is often prohibited by methylation in the hTERT promoter. In two studies with breast-cancer cell lines, EGCG or an EGCG prodrug with enhanced stability and bioavailability reduced promoter methylation at selected CpG sites in the hTERT promoter. This facilitated E2F binding, as measured by chromatin immunoprecipitation (ChIP), and led to reduced hTERT mRNA expression and reduced cell proliferation (Berletch *et al.*, 2008; Meeran *et al.*, 2011).

Table 6.5 Influence of (−)-epigallocatechin gallate (EGCG) and green-tea polyphenols (GTPs) on DNA methylation.

Mechanism/target	Effect	Cell line/tissue	Genes/factors analyzed	References
DNA methylation *in vitro*				
In silico modeling		Homology model of the catalytic domain of hDNMT1	Hydrogen bonding with Glu1265, Ser1229, and Arg1309 in the catalytic pocket of DNMT	Fang *et al.* (2003), Lee *et al.* (2005), Yoo & Medina-Franco (2011)
DNMT activity *in vitro*	Inhibition	KYSE 510 nuclear extracts	IC_{50}: 20 µM	Fang *et al.* (2003),
		M.Sss1 bacterial enzyme	IC_{50}: 0.2 µM	Lee *et al.* (2005)
		rec. DNMT1	IC_{50}: 0.5 µM	
	Inhibition	rec. DNMT1	Considered an artifact due to unspecific enzyme inhibition	Medina-Franco *et al.* (2011)
DNMT activity in cell culture	Reduction	LNCaP nuclear extracts		Pandey *et al.* (2009)
	Reduction	A431		Nandakumar *et al.* (2011)
	Reduction	MCF7, MDA-MB-231		Meeran *et al.* (2011, 2012)
DNMT mRNA/protein expression	Reduction (GTP)	LNCaP	DNMT1	Pandey *et al.* (2009)
	Reduction	A431	DNMT1, DNMT3a, DNMT3b	Nandakumar *et al.* (2011)
	Reduction	Jurkat	DNMT1, DNMT3a, DNMT3b	Wong *et al.* (2011)
	No effect	KYSE 510	DNMT1, DNMT3a, DNMT3b, MBD2	Fang *et al.* (2003)
Global methylation	Reduction	A431, Jurkat	Genomic 5meC content	Nandakumar *et al.* (2011), Wong *et al.* (2011)
	No effect	T24, HT29, PC3 TK6, HCT116	MAGE-A1, Alu, LINE repetitive elements, genomic 5meC content	Chuang *et al.* (2005), Stresemann *et al.* (2006)
	Increase (GTP)	LNCaP	LINE-1 repetitive elements	Pandey *et al.* (2009)
Promoter hypermethylation	Reduction	KYSE 510	p16, RARβ, MGMT, hMLH1, associated with gene re-expression	Fang *et al.* (2003, 2007)
	Reduction	HT29, Caco-2	p16 (via folate metabolism)	Fang *et al.* (2003), Navarro-Peran *et al.* (2007)

(*continued overleaf*)

Table 6.5 (*continued*)

Mechanism/target	Effect	Cell line/tissue	Genes/factors analyzed	References
	No effect	T24, HT29, PC3	p16, also no effect on gene expression	Chuang *et al.* (2005)
	Reduction	PC3, KYSE 150, MCF7, MDA-MB-231	RARβ	Fang *et al.* (2003), Lee *et al.* (2005)
	Reduction	MCF7, MDA-MB-231	hTERT, associated with reduced gene expression	Berletch *et al.* (2008), Meeran *et al.* (2011)
	Reduction	HSC3, HSC4, SCC9, SCC25	RECK, associated with gene re-expression, ↓ invasive potential	Kato *et al.* (2008)
	Reduction	H460, A549	WIF-1, associated with gene re-expression	Gao *et al.* (2009)
	Reduction (GTP)	LNCaP	GSTP1	Pandey *et al.* (2009)
mRNA/protein re-expression	Induction	KYSE 510, CL13	p16, RARβ, DAPK	Fang *et al.* (2007)
	Induction	A431	p16, p21	Nandakumar *et al.* (2011)
	Induction (GTP)	LNCaP	GSTP1	Pandey *et al.* (2009)
	Induction (EGCG, GTP)	Jurkat	Foxp3	Wong *et al.* (2011)
DNA methylation *in vivo*				
SAM levels; SAM:SAH ratio	Moderate reduction	Plasma, small intestine, liver in healthy mice		Fang *et al.* (2007)
SAH, methionine, homocysteine level	No effect	Plasma, small intestine, liver in healthy mice		Fang *et al.* (2007)
Global methylation	No effect	Prostate, gut, liver in TRAMP mice	Global 5mC level; B1 repetitive elements, MAGE-a8	Morey Kinney *et al.* (2009)
Promoter methylation	Reduction	Colon, small intestine, AOM-treated $APC^{Min/+}$ mice	RXRα	Volate *et al.* (2009)
	No effect	Prostate, gut, liver in TRAMP mice	IRX3, CACNA1A, CDKN2A, NRX2	Morey Kinney *et al.* (2009)
	Reduction	Gastric-cancer patients	CDX2,BMP2	Yuasa *et al.* (2009)
	No effect	Gastric-cancer patients	p16, CACNA2D3, GATA5, ER	Yuasa *et al.* (2009)

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a novel tumor-suppressor gene that inhibits matrix-metalloproteases. Kato *et al*. (2008) treated oral squamous cell carcinoma cell lines with EGCG and detected reduced promoter methylation, accompanied by mRNA re-expression of RECK. This led to reduced levels and activities of metalloproteases, and invasive potential through collagen matrices was inhibited.

Disruption of the Wnt signaling pathway and aberrant activation of the transcriptional coactivator β-catenin play an important role in cancer development in various organs, including colon, skin, liver, ovary, breast, and lung (Barker & Clevers, 2006). WIF1 is an endogenous antagonist of the Wnt signaling pathway that is frequently disrupted by DNA methylation (Klaus & Birchmeier, 2008). In lung-cancer cell lines, EGCG treatment enhanced WIF1 mRNA expression by lowering promoter methylation levels. This was associated with reduced β-catenin expression and inhibition of Wnt signaling in a reporter assay (Gao *et al.*, 2009).

Glutathione S-transferase P1 (GSTP1) is frequently silenced in prostate cancer by promoter hypermethylation. In a study by Pandey *et al.* (2009), inhibition of DNMT activity in LNCaP prostate cancer cells by GTP reduced GSTP1 promoter methylation and led to its re-expression. ChIP experiments indicated reduced MBD2 (methyl-CpG binding domain protein 2) binding to transcription factor Sp1 (specificity protein 1) binding sites and induced transcriptional activation of the GSTP1 promoter.

Wong *et al.* (2011) investigated the effect of EGCG and GTP on FoxP3 methlyation and expression. FoxP3 controls the development and function of regulatory T cells (Treg). Both interventions reduced FoxP3 promoter methylation and led to significant up-regulation of FoxP3 mRNA expression.

Taken together, these data demonstrate that EGCG and GTP not only inhibit DNMT activity *in vitro* but also reduce promoter methylation in cultured cells, leading often to gene re-expression. So far, these studies have focused on selected candidate genes. Consequently, it is presently not known whether EGCG affects only selected genes or has a global reactivating effect on genes silenced by promoter methylation. One question that also needs to be answered is whether EGCG-mediated gene reactivation is relevant for its cancer-preventive efficacy.

Studies investigating the influence of EGCG on DNA methylation in rodent models and in human epidemiological studies have so far produced inconsistent results (Table 6.5). Fang *et al.* (2007) reported a moderate reduction of SAM levels and of the SAM : SAH ratio in various tissues and plasma of healthy mice, without an effect on SAH, methionine, or homocysteine levels. Morey Kinney *et al.* (2009) analyzed the prostate cancer preventive potential of GTP in "TRansgenic Adenocarcinoma of Mouse Prostate" (TRAMP) mice and used a genome-wide approach to identify genes with altered DNA methylation in prostate, gut, and liver. Although earlier studies had repeatedly reported good prostate-cancer preventive activity of GTP in this model (Gupta *et al.*, 2001; Caporali *et al.*, 2004; Adhami *et al.*, 2009), Morey Kinney *et al*. (2009) could not demonstrate any change in global me5C levels, any locus-specific or genome-wide DNA methylation, or any preventive effect on prostate cancer development.

$APC^{Min/+}$ mice develop multiple adenomas in the small intestine due to a genetic defect in the Wnt signaling pathway. Additional treatment with the carcinogen azoxymethane induces development of colonic tumors, which are generally rare in the $APC^{Min/+}$ model. Volate *et al*. (2009) treated $APC^{Min/+}$ mice and wt controls with GTP in drinking water for 4–8 weeks. Numbers of both small-intestinal adenomas and colon tumors were significantly reduced: by 28 and 50%, respectively. Also, GTP prevented AOM-induced promoter methylation and silencing of RXRα in colonic mucosa. RXRα expression is often reduced in human colon cancer. Therefore, these data might be of relevance for human colon cancer prevention.

In a human intervention study with 106 gastric-cancer patients, high consumption of green tea (≥7 cups per day) significantly lowered the frequency of promoter methylation of the homeobox transcription factor CDX2 (caudal type homeobox 2) and bone morphogenetic protein BMP2 in primary tumor samples, whereas methylation of p16 (INK4A), calcium channel-related CACNA2D3 (calcium channel, voltage-dependent, alpha 2/delta subunit 3), transcription factor GATA-5 (GATA binding protein 5, binding to the DNA sequence GATA), and estrogen receptor (ER) was not changed (Yuasa *et al.*, 2009).

So far, the reason why EGCG inhibits or prevents methylation at certain promoter regions while others genes are not affected has not been investigated in detail. In principle, a sequence-specific effect on DNA methylation is feasible. It is well documented that transcription factors recruit activating or repressing complexes containing DNMTs to the DNA (Robertson *et al.*, 2000; Rountree *et al.*, 2000; Di Croce *et al.*, 2002). This is additionally regulated in a cell type-specific manner by the abundance of coactivators and corepressors. A further layer of regulation is the CpG density and the level of DNA methylation, as well as the regulation of DNA accessibility by the chromatin substructure at a certain DNA region. As long as genome-wide DNA methylation information is not available and interpretations are limited to a number of candidate genes, this question cannot be conclusively answered.

6.5.1.2 Histone-modifying enzymes (HATs, HDACs, HMTs)

Since 2009, several studies have revealed that the epigenetic effects of EGCG and GTP are not limited to influencing DNA methylation. As summarized in Table 6.6, EGCG has been shown to enhance histone acetylation by both *enhancing* p300 HAT expression and activity and *reducing* HDAC expression and activity in cultured cells. This leads to enhanced expression of ERα and reduced expression of hTERT in breast-cancer cells (Li *et al.*, 2010c; Meeran *et al.*, 2011, 2012), and to induction of the cell-cycle regulators p16 and p21 in lung-cancer cells (Nandakumar *et al.*, 2011). In LNCaP cells, reduced expression of HDAC isoforms enhanced acetylation and activation of p53 as a non-histone HDAC substrate. As a consequence, expression of p21 and the pro-apoptotic Bax were induced, leading to cell-cycle arrest and induction of apoptosis (Thakur *et al.*, 2012a,b).

Several studies have reported an *inhibitory effect* of EGCG on HAT activity, mainly p300, associated with reduced acetylation levels of histone H3 and H4, as well as of the non-histone HAT substrates NF-κB p65 and androgen receptor (AR). As a consequence, NF-κB- and AR-mediated cell signaling were reduced by EGCG treatment. The activities

Table 6.6 Influence of (−)-epigallocatechin gallate (EGCG) and green-tea polyphenols (GTPs) on histone-modifying enzymes.

Mechanism/target	Effect	Cell line/tissue	Genes/factors analyzed	References
HAT activity HDAC activity	Induction Reduction	MDA-MB-231	↑ p300 expression ↓ HDAC1 expression ↑ ac-H3, H3K9ac, ac-H4 ↓ HMT SUV39H1 expression ↑ H3K4me2; ↓ H3K9me3 ↑ ERα reactivation	Li *et al.* (2010c), Meeran *et al.* (2012)
HDAC activity	Reduction	A431	↑ ac-H3, ac-H4 ↑ p16, p21 protein expression	Nandakumar *et al.* (2011)
	Reduction (prodrug)	MCF7, MDA-MB-231	↑ ac-H3, H3K9ac ↑ binding of MAD1, E2F1 to hTERT promoter; ↓ binding of c-Myc to hTERT promoter ↓ hTERT expression	Meeran *et al.* (2011)
	Reduction	LNCaP, PC3	↓ HDAC1, 2, 3, 8 protein expression ↑ p53 acetylation and activation ↑ p21, Bax expression ↑ cell cycle arrest, apoptosis	Thakur *et al.* (2012a,b)
HAT activity: p300, PCAF, Tip60	Reduction	HeLa, Hek293	↔ SIRT1, HDAC, HMT activity ↓ ac-H3, ac-H4 ↓ NF-κB p65 acetylation ↑ cytosolic IκBα ↓ p65-binding to IL6 promoter, ↓ NF-κB-target gene expression ↓ EBV-mediated IL-6, IL-12 expr ↓ EBV-mediated cell transformation	Choi *et al.* (2009a)

(*continued overleaf*)

Table 6.6 (*continued*)

Mechanism/target	Effect	Cell line/tissue	Genes/factors analyzed	References
		TNFα-stimulated mice	↓ serum IL-6 ↓ p65 acetylation in macrophages ↓ cytokine expression	Choi *et al.* (2009a)
HAT activity: p300	Reduction	LNCaP	↓ ac-H3	Lee *et al.* (2012)
SIRT1, HDAC, HMT activity	No effect		↓ AR acetylation, nuclear translocation ↓ AR-mediated PSA, NKX3.1 mRNA expression ↓ cell growth	
HMT protein expression: BMI-1, SUZ12, EZH2, EED, Mel18	reduction	SCC-13, HaCaT, A431	↓ H3K27me3 ↑ p21 and p27 expression ↑ cell cycle arrest ↑ apoptosis BMI over-expression reverses the effects of EGCG	Balasubramanian *et al.* (2010), Choudhury *et al.* (2011)

of other histone-modifying enzymes (SIRT1, HDAC, HMT) were not inhibited by the polyphenol (Choi *et al.*, 2009a; Lee *et al.*, 2012).

A recent study suggests that EGCG also targets histone methylation (Balasubramanian *et al.*, 2010). The polycomb group (PcG) proteins BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog) and EZH2 regulate gene expression by increasing the repressive H3K27 methylation mark and reducing histone acetylation. EGCG reduced the expression of BMI-1 and EZH2 in skin-cancer cells, which was consistent with reduced H3K27me3 levels. Also, EGCG enhanced the expression of p21 and p27 cell-cycle regulators and induced apoptosis. Over-expression of BMI-1 reversed the effects of EGCG, indicating that inhibition of BMI-1-mediated H3K27 methylation contributes to the cell-growth inhibitory effect of EGCG (Balasubramanian *et al.*, 2010; Choudhury *et al.*, 2011).

6.5.1.3 miRNAs

The development of microarray-based approaches has facilitated investigations of natural product-mediated effects on miRNAs expression, similar to gene-expression studies. To avoid over-interpretation, the results of microarray-based studies should be verified by RT-PCR or by over-expression/antagonization of altered miRNAs and measurement of the expression of their target genes. The present review is therefore limited to those miRNA screening results obtained with EGCG, for which alterations in miRNA expression were confirmed by alternative methods (Table 6.7).

Tsang & Kwok (2010) investigated the effect of EGCG on miRNA expression in HepG2 human hepatocellular carcinoma cells. miR-16, let-7a, and miR-221 were up-regulated, whereas miR-18a, miR34b, miR-193, miR-222, and miR-342 were down-regulated. One of the targets of miR-16 is the anti-apoptotic protein Bcl-2. The authors could confirm that EGCG treatment reduced expression of Bcl-2 and induced apoptosis in HepG2 cells. Data were verified by transfection with a miR-16 inhibitor (Tsang & Kwok, 2010). In MCF7 breast-cancer cells, intervention with a GTP extract, polyphenon-60, up-regulated miR-548m and miR-720 expression. These data were confirmed by RT-PCR analyses. Concomitantly, miR-27a was down-regulated, but this finding could not be verified (Fix *et al.*, 2010). The influence of polyphenon-60 on miRNA target genes was not investigated.

Wang *et al.* (2011a) analyzed the effect of EGCG on proliferation of mouse and human lung-cancer cells and investigated regulation of miRNAs as an underlying mechanism. HIF-1α (hypoxia-inducible factor 1-alpha), a major transcription factor involved in angiogenesis, is unstable under normoxic conditions and is stabilized during hypoxia. Over-expression of miR-210, induced by EGCG, was found to be associated with stabilization of HIF-1α, which was shown to regulate miR-210 expression (Wang *et al.*, 2011a).

In a prostate-cancer xenograft model, treatment of mice with EGCG reduced tumor growth and resulted in up-regulation of the tumor suppressor miR-330 and down-regulation of androgen-responsive miR-21 (Siddiqui *et al.*, 2011). Molecular modeling and functional studies indicate that EGCG binds to the AR and disrupts androgen signaling.

In a recent study, EGCG in combination with a retinol derivative inhibited the growth of human malignant neuroblastoma cells. Expression of the tumor-suppressor miRs miR-7-1, miR-34a, and miR-99a was increased by the combination, whereas expression of oncogenic

Table 6.7 Influence of (−)-epigallocatechin gallate (EGCG) and green-tea polyphenols (GTPs) on miRNA expression (verified by RT-PCR).

Mechanism/target	Effect	Cell line/tissue	Genes/factors analyzed	References
miR-16, let-7a, miR-221	Up-regulation	HepG2	↓ Bcl-2 by miR-16 confirmed by precursor and inhibitor transfection	Tsang & Kwok (2010)
miR548m, miR-720	Up-regulation (GTP)	MCF7		Fix *et al.* (2010)
miR-210	Up-regulation	H1299 CL13 (mouse lung)	Mediated via HIF-1 stabilization ↓ cell growth	Wang *et al.* (2011a)
miR-330	Up-regulation	C4-2 and 22Rv1prostate xenografts	Mediated via reduced AR signaling	Siddiqui *et al.* (2011)
miR-7-1, miR-34a, miR-99a	Up-regulation	SK-N-BE2 IMR-32	Over-expression of miR-7-1, augmenting induction of apoptosis	Chakrabarti *et al.* (2012)
miR-18a, miR-34b, miR-193b, miR-222, miR-342	Reduction	HepG2		Tsang & Kwok (2010)
miR-27a	Reduction (GTP)	MCF7		Fix *et al.* (2010)
miR-21	Reduction	C4-2 and 22Rv1prostate xenografts	Mediated via reduced AR signaling	Siddiqui *et al.* (2011)
miR-92, miR-93, miR-106b	Reduction	SK-N-BE2 IMR-32	Over-expression of miR-93, counteracting induction of apoptosis	Chakrabarti *et al.* (2012)

miR-92, miR-93, and miR-106b was down-regulated. Over-expression of miR-7-1 and miR-93 augmented and counteracted the effects of the EGCG and retinamide combination.

Overall, these data indicate that alterations in the expression of miRNAs need to be considered when EGCG treatment modulates expression of miRNA target genes. The mechanism by which specific miRNAs are up-regulated or repressed by EGCG treatment is presently not known.

6.5.2 Genistein and soy isoflavones

Isoflavones are a class of flavonoids abundantly found in plants of the *Fabaceae* family and characterized by phytoestrogenic properties. The isoflavones genistein and daidzein are contained in high concentrations in soybean (*Glycine max* L.) and are enriched in soy products. Epidemiological evidence indicates an inverse correlation between a traditional soy-rich, low-fat Asian diet and the risk of developing breast and prostate cancer (Messina *et al.*, 2006; Jian, 2009; Magee & Rowland, 2012). As phytoestrogens, soy isoflavones bind to the estrogen receptor and modulate ER signaling. Genistein has been shown to affect several additional chemopreventive mechanisms, including activation of carcinogens, inhibition of oxidative stress, inflammation, cell signaling, angiogenesis, modulation of cell-cycle regulation, and induction of apoptosis (Steiner *et al.*, 2008; Molinie & Georgel, 2009; Magee & Rowland, 2012). Preventive efficacy against breast, ovarian, prostate, skin, stomach, and colon cancer has been demonstrated in various rodent models (Banerjee *et al.*, 2008). Recent observations of a growth-promoting effect of genistein in ER-positive breast-cancer cell lines and xenograft models have indicated a potential risk of genistein for human health (Helferich *et al.*, 2008; Rietjens *et al.*, 2013). A recent review does not support these concerns (Magee & Rowland, 2012). Genistein is currently being tested in various clinical trials for the treatment and prevention of prostate, bladder, kidney, breast, and endometrial cancer (Taylor *et al.*, 2009).

6.5.2.1 DNA methylation

Several studies have identified genistein as a polyphenol that affects epigenetic mechanisms at the level of DNA methylation (Table 6.8), modulation of histone marks, and miRNA expression. Fang *et al*. (2005) identified genistein as a dose-dependent inhibitor of DNMT activity with both nuclear extracts derived from human esophageal cells and human recombinant DNMT1. Genistein was also found to reduce DNMT activity in various cell lines; this was at least partly due to reduced expression of all three DNMTs: 1, 3a, and 3b (Li *et al.*, 2009a; Majid *et al.*, 2009). Fang *et al.* (2005, 2007) further reported that genistein reduced promoter methylation and led to re-expression of RARβ, the cell-cycle inhibitor p16, and the DNA repair gene MGMT in KYSE 510 esophageal cancer cells. RARβ demethylation associated with re-expression was also seen in the KSYE 150 esophageal and two prostate cancer cell lines in a time- and dose-dependent manner (Fang *et al.*, 2005), as well as in HCT116 colon and SiHa squamous cervical-cancer cells (Spurling *et al.*, 2008), but not in two breast-cancer cell lines (King-Batoon *et al.*, 2008).

Table 6.8 Effects mediated by genistein and soy isoflavones on DNA methylation.

Mechanism/target	Effect	Cell line/tissue	Genes/factors analyzed	References
DNA methylation *in vivo*				
DNMT activity *in vitro*	Inhibition	KYSE 510 nuclear extracts, human recombinant DNMT1		Fang *et al.* (2005)
DNMT activity in cell culture	Reduction	A498, ACHN, HEK-293 nuclear extracts		Majid *et al.* (2009)
DNMT mRNA/protein expression	Weak reduction	A498, ACHN, HEK-293	DNMT1, DNMT3a, DNMT3b	Majid *et al.* (2009)
	Reduction	MCF7, (MCF10AT)	DNMT1, DNMT3a, DNMT3b	Li *et al.* (2009a)
Promoter hypermethylation	Reduction	KYSE 510	RARβ, p16, MGMT, associated with gene re-expression	Fang *et al.* (2005, 2007)
	Reduction	PC3, LNCaP, KYSE 150	RARβ, associated with gene re-expression	Fang *et al.* (2005)
	Reduction	HCT116, SiHa	RARβ2, associated with gene re-expression	Spurling *et al.* (2008), Jha *et al.* (2010)
	No effect	MCF7, MDA-MB-468	RARβ	King-Batoon *et al.* (2008)
	Reduction	MDA-MB-468	GSTP1, associated with gene re-expression	King-Batoon *et al.* (2008)
	No effect	MCF7	GSTP1	King-Batoon *et al.* (2008)
	Reduction	DU-145, PC-3, LNCaP	GSTP1, EPHB2	Vardi *et al.* (2010)
	No effect	DU-145, PC-3, LNCaP	RASSF1A, BRCA1	Vardi *et al.* (2010)
	Reduction	DU-145, PC-3	GSTP1, EPHB2, BRCA, associated with gene re-expression	Adjakly *et al.* (2011)
	Reduction	SW1116	Wnt5a, associated with gene re-expression	Wang & Chen (2010)
	Reduction	MCF10AT, MCF7	hTERT (E2F-1 binding site), associated with reduced expression	Li *et al.* (2009a)
	Reduction	A498, ACHN, HEK-293, LNCaP, PC-3, RWPE-1	BTG, associated with gene re-expression in cancer cell lines	Majid *et al.* (2009, 2010b)
	Reduction	Mouse embryonic stem cells	Ucp1, Sytl1	Sato *et al.* (2011)
	Reduction	AGS	PCDH17, associated with gene ge-expression	Yang *et al.* (2012)
	Reduction	MCF7, MDA-MB-231, MCF10a	BRCA1, BRAC2, associated with weak protein re-expression, also seen with the daidzein metabolite equol	Bosviel *et al.* (2012a,b)

DNA methylation ***in vivo***	Increase or no effect	Pancreas, rats treated neonatally with equol (daidzein metabolite)	Oncogenic Ha-Ras, no effect on c-Myc, c-Fos	Lyn-Cook *et al.* (1995)
	Increase	prostate, healthy male mice, up to 4 weeks' intervention	Specific clones (no gene specified)	Day *et al.* (2002)
	Increase	*In utero* exposure, A^{vy} (Agouti) mice	A^{vy} intracisternal A particle (IAP) murine retrotransposon	Dolinoy *et al.* (2006)
	No effect	*In utero* exposure, Agouti mice	Coat color	Badger *et al.* (2008)
	No effect	*In utero* exposure, Agouti mice	Coat color	Rosenfeld *et al.* (2013)
	Increase	*In utero* exposure, mice	Repetitive elements in bone marrow, down-regulation of estrogen-responsive genes	Vanhees *et al.* (2011)
	Increase	PBMC of heavy smokers, 4 weeks' intervention with soy and green tea	Line1 repetitive elements	Scoccianti *et al.* (2011)
	Reduction	Uterus in healthy mice, intact and ovarextomized (OVX)	Nsbp1	Tang *et al.* (2008a)
	Reduction	Pancreas, liver in healthy mice	ActaI	Guerrero-Bosagna *et al.* (2008)
	Reduction	Endometrium, ovariectomized (OVX) mice	SF-1, associated with re-expression	Matsukura *et al.* (2011)
	Reduction or increase	Cynomolgus monkeys	Fat tissue: ↓ABCG5, ↑TBX5, ↑HoxB1 Muscle: ↑HoxA5, ↑HoxA11, ↓NTRK3	Howard *et al.* (2011)
	Reduction	Healthy premenopausal women, intraductal breast specimens	RARβ2, CCDN2 (in women with low circulating serum genistein levels)	Qin *et al.* (2009)
	No effect	Healthy premenopausal women, intraductal breast specimens	ER, p16, RASSF1A	Qin *et al.* (2009)
	No effect	PBMC of heavy smokers, 4 weeks' intervention with soy and green tea	RASSF1A, ARF, CDKN2, MLH1, MTHFR	Scoccianti *et al.* (2011)

Methylation at the GSTP1 promoter was reduced and the gene was weakly re-expressed after repetitive treatments with genistein only in ER-negative, Her2-overexpressing MDA-MB-468 breast-cancer cells, whereas similar treatment was ineffective in reactivating GSTP1 in ER-positive MCF-7 cells (King-Batoon *et al.*, 2008). However, genistein and daidzein intervention significantly reduced GSTP1 promoter methylation in the prostate-cancer cell lines LNCaP, PC3, and DU-145, leading to re-expression of GSTP1 protein, as determined by immunocytochemistry and Western blotting (Vardi *et al.*, 2010; Adjakly *et al.*, 2011).

In a study by Vardi *et al.* (2010), methylation of two other genes often silenced by promoter hypermethylation in cancer, the candidate tumor suppressor gene RASSF1A (Ras association domain family 1, isoform A) and the DNA repair gene BRCA1 (breast cancer 1, early-onset), was not reduced in prostate-cancer cell lines; Adjakly *et al.* (2011) could demonstrate BRCA and EPHB2 (ephrin receptor B2) demethylation and re-expression in DU-145 and PC-3 prostate cancer cells, on the other hand. Consistently, genistein and daidzein treatment, as well as the daidzein metabolite equol, led to demethylation and re-expression of BRCA1 and 2 protein in three breast (cancer) cell lines: MCF-7, MDA-MB-231 and MCF 10a (Bosviel *et al.*, 2012a,b).

Wnt5a is a ligand of the Wnt receptor that is silenced by promoter methylation in colon-cancer cell lines. Genistein treatment of SW1116 colon-cancer cells reduced cell viability by 80%. Concomitantly, Wnt5a promoter methylation decreased by about 10% in association with an increase of Wnt5a mRNA levels (Wang & Chen, 2010).

Hypomethylation at the hTERT core promoter facilitates binding of the repressor E2F-1 and reduces hTERT expression. In the breast-cancer cell lines MCF7 and MCF10AT, genistein-mediated down-regulation of DNMT expression was associated with reduced hTERT methylation and reduced expression (Li *et al.*, 2009a).

The tumor suppressor BTG3 (B cell translocation gene 3) is a negative regulator of cell signaling and inhibits E2F. Genistein intervention in renal- and prostate-cancer cell lines reduced promoter methylation and increased BTG3 mRNA levels. This was further facilitated by genistein-mediated effects on HDAC and HAT activity, in association with enhanced binding of acetylated and methylated histones to the BGT promoter, indicating increased transcription (Majid *et al.*, 2009, 2010b).

Using a mouse embryonic stem (ES) cell differentiation system as a model, Sato *et al.* (2011) analyzed the influence of genistein on epigenetic reprogramming during fetal development. Genome-wide methylation analyses indicated a moderate effect on DNA methylation in a subset of genes. Ucp1 and Sytl1 promoters were selected for focused analyses of methylation dynamics. Methylation at selected CpG sites was reduced by genistein treatment after initial *de novo* methylation, which was not affected by genistein treatment. Also, differentiation of ES cells to contracting cardiomyocytes was not affected by genistein (Sato *et al.*, 2011).

PCDH17 (protocadherin 17) belongs to the cadherin protein family (Yang *et al.*, 2012). In the gastric-cancer cell line AGS, expression of PCDH17 was found to be reduced by promoter methylation. Treatment with genistein lowered methylation levels and reactivated PCDH17 expression to a similar extent as seen with the demethylating agent

5-aza-2′-deoxycytidine. It also induced cell-cycle arrest and reduced cell proliferation (Yang *et al.*, 2012).

As indicated before, analyses of the demethylating effects of genistein in cell culture are not totally conclusive. This may be a result of the varying dose range, cell culture-, and compound-treatment conditions, as well as of differences in the methodology used to determine DNA methylation. Although metabolism of isoflavones differs between rodents and humans, modulation of epigenetic effects in rodent models might be a more reliable indicator for activity in humans than the results of *in vitro* cell-culture studies.

An early study published by Lyn-Cook *et al*. (1995) used methylation-sensitive restriction digestion to compare fragmentation patters of the c-Ha-Ras oncogene in rat pancreas after neonatal intervention with the daidzein metabolite equol (100 μg) and another phytoestrogen, coumestrol (10 μg), for 10 days. Southern blotting indicated increased methylation at the c-Ha-Ras gene, but not at c-Myc of Fos proto-oncogenes.

Day *et al.* (2002) applied genistein at a dose of 300 mg/kg diet to male mice for up to 4 weeks. Changes in DNA CGI methylation in the prostate were analyzed by differential methylation hybridization after digestion of DNA with methylation-sensitive restriction enzymes. Methylation of three DNA fragments increased after 4 weeks' genistein intervention; no methylation changes were detected in liver DNA. Dolinoy *et al.* (2006) investigated the effect of *in utero* exposure to genistein in the Agouti mouse model. Expression of the Agouti gene responsible for coat color was epigenetically regulated by a retrotransposon upstream of its transcription start site. An increase in methylation at six CpG sites in the retrotransposon was detected in the offspring of female mice exposed to dietary genistein (250 mg/kg diet) during gestation, and coat color was shifted towards pseudoagouti, indicative of reduced expression of the Agouti gene. Two later studies did not observe coat-color changes following *in utero* genistein exposure (Badger *et al.*, 2008; Rosenfeld *et al.*, 2013). A study by Vanhees *et al.* (2011) indicated that prenatal maternal exposure of mice to genistein (270 mg/kg diet) was associated with hypermethylation at repetitive elements in bone marrow and down-regulated ER-responsive genes in hematopoeietic cells of the offspring.

Methylation at repetitive elements can be interpreted as a sign of genomic stability. Scoccianti *et al*. (2011) performed a 4-week intervention study in heavy smokers with diets enriched in cruciferous vegetables or supplemented with flavonoids in the form of soy products and green tea. They compared methylation of the LINE-1 (long interspersed nuclear element) repetitive element and of a series of genes known as tumor-suppressor genes, involved in DNA repair and folate metabolism in DNA derived from peripheral white blood cells (PBMCs) collected before and after intervention. Both diets slightly but significantly increased methylation of LINE-1, by 0.8 and 2.1% respectively, whereas methylation of selected candidate genes such as RassF1A, the cell-cycle regulators ARF (alternate reading frame of the INK4a/ARF locus CDKN2A) and CDKN2A (cyclin-dependent kinase inhibitor 2A, also known as p16), the DNA repair gene MLH, and MTHRF (methylenetetrahydrofolate reductase) with a major role in folate metabolism did not change significantly (Scoccianti *et al.*, 2011). There is an ongoing debate over whether PBMC can be used as surrogate tissue to identify physiologically relevant changes in DNA methylation (Wang *et al.*, 2010; Brennan *et al.*, 2012). Notably, analyses can be confounded by variation in

white blood cell composition (Adalsteinsson *et al.*, 2012; Houseman *et al.*, 2012; Reinius *et al.*, 2012).

The influence of genistein or daidzein exposure on gene-promoter methylation was investigated in several rodent models (Guerrero-Bosagna *et al.*, 2008; Tang *et al.*, 2008a; Matsukura *et al.*, 2011), a study on macaques (Howard *et al.*, 2011), and in breast specimens of healthy women (Qin *et al.*, 2009). In a study by Tang *et al.* (2008a), mice were neonatally exposed to genistein (50 mg/kg bodyweight, b.w.) or diethylstilbestrol (DES) at three doses by subcutaneous injection at days 1–5. At the age of 6 months, uterine DNA was extracted and differentially methylated regions were identified by methylation-sensitive restriction fingerprinting. Five hyper- and five hypomethylated regions mostly localized in intronic CGIs were identified after genistein treatment; four additional regions showed a modulation of DNA methylation after treatment with DES at the highest dose (1 mg/kg b.w.). A region associated with a promoter CGI of nucleosomal binding protein 1 (Nsbp1) that was hypomethylated after DES treatment was selected for further analysis. Nsbp1 plays an important role in chromatin remodeling. Neonatal DES/genistein exposure prevented hypermethylation of the Nsbp1 promoter during puberty and aging in intact animals, but not in ovarectomized mice, and led to its continuous expression (Tang *et al.*, 2008a).

In a mouse study by Guerrero-Bosagna *et al*. (2008), dietary soy exposure *in utero* (2% in the parental diet, starting 2 weeks before mating) led to accelerated vaginal opening in female offspring, indicating physiological activity. The intervention did not modulate DNA methylation levels of skeletal α-actin (Acta1) in the liver and pancreas of male and female offspring, but reduced gender differences in methylation levels. Exposure of ovarectomized mice to high- (200 kg/kg diet) and low-dose (60 mg/kg diet) genistein for 1 week reduced methylation and induced expression of steroidogenic factor 1 (SF-1, gene name NR5A1) in endometrial cells. SF-1 is an orphan nuclear receptor and transcription factor for key enzymes involved in steroidogenesis (Matsukura *et al.*, 2011).

Howard *et al.* (2011) were interested in whether typical commercial animal chows based on soy protein and rich in soy isoflavones would modulate DNA methylation patterns in various tissues of non-human primates. A total of eight cynomolgus macaques were assigned to two high-fat diets based on either casein or soy (equivalent to 180 mg soy isoflavones for a human adult per day) as the protein source, with similar macronutrient contents, for 8 weeks in a cross-over design. HumanMethylation 27k arrays (Illumina) were used to determine genome-wide methylation changes in subcutaneous adipose tissue, muscle, and liver. A switch from a soy-based to a casein-based diet resulted in a general increase in methylation levels in liver and muscle tissue. Specifically, in muscle tissue two homeobox genes, HOXA5 and HOXA11, with functions as developmental transcription factors were demethylated during the switch from the soy- to the casein-based diet, whereas methylation at the promoter of the NTRK3 gene (neurotrophic tyrosine kinase, receptor, type 3) increased. In fat tissue, the switch from the casein- to the soy-based diet resulted in reduced methylation at the ABC-transporter G5 (ABCG5) and increased methylation at the promoters of HOXB1 and T-box protein 5 (TBX5). T-box proteins share a T-box-binding domain; they are transcription factors with important functions in the regulation of developmental processes. Methylation changes for these genes in fat tissue were confirmed by pyrosequencing (Howard *et al.*, 2011). In addition to changes in methylation, the diet switch from

casein to soy resulted in reduced fasting insulin levels and improved insulin sensitivity. The same group had shown earlier that maintaining the animals on a soy-based diet for up to 2 years led to reduced body weight and improved blood lipids compared to those on the casein-based diet. Qin *et al.* (2009) performed a 4-week dietary intervention in 34 healthy premenopausal women with daily doses of 40 or 140 mg isoflavones. They reported dose-dependent changes in RARβ2 and CCND2 (cyclin D2) promoter methylation in mammary tissue in women with low circulating serum genistein levels, whereas the intervention did not influence Rassf1A, ER, or p16 methylation in intraductal breast specimens (Qin *et al.*, 2009).

Although these results are promising, so far a direct relation between modulation of DNA methylation by genistein and chemopreventive potential *in vivo* has not been demonstrated.

6.5.2.2 Influence on histone acetylation and methylation

In addition to its effects on DNA methylation, genistein has been reported to affect acetylation and methylation of histones and non-histone proteins, and as a consequence to alter gene expression (Table 6.9). Several studies have focused on effects related to breast-cancer prevention. Genistein and other phytoestrogens are known to interact with ER-α and -β. After ligand binding, the ER dimer recruits nuclear coactivators and the complex interacts with estrogen-responsive elements in the promoter regions of estrogen-regulated genes. In contrast, anti-estrogen binding to the ER results in the recruitment of corepressors. Coactivators often have HAT activity, whereas corepressors possess HDAC activity (Powles, 2002). In a test-tube experiment with isolated ER-β, coactivators, chromatin, and [^{3}H]-labeled acetyl-CoA as a substrate, genistein, daidzein, and equol stimulated ER-β-mediated histone acetylation (Hong *et al.*, 2004). In ER-positive MCF-7 breast-cancer cells, long-term treatment for 40–60 days with low-dose genistein at a concentration of 10 nM reduced basal histone H3 acetylation and desensitized the cells towards the HDAC inhibitors TSA and apicidin. Also, cell proliferation and the mitogenic response to estradiol and epidermal growth factor were reduced, suggesting that long-term genistein treatment might lead to epigenetic changes supporting cancer-preventive potential (Jawaid *et al.*, 2010).

A recent study by Li *et al.* (2013b) indicates that genistein can reactivate ER-α expression in ER-negative cell lines such as MDA-MB-231 and resensitize these cells to treatment with anti-estrogens such as tamoxifen via a histone acetylation-related mechanism, especially in combination with TSA. Accordingly, enhanced tumor-growth inhibition by dietary genistein (250 mg/kg) in combination with tamoxifen was demonstrated *in vivo* in a MDA-MB-231 xenograft model and in the C3(1) SV40 TAg transgenic mouse model for basal breast cancer. This combined treatment may have implications for the clinical management of hormone receptor-negative breast cancer (Li *et al.*, 2013b). Li *et al.* (2013a) also used human mammary epithelial cells (HMEC) transformed with SV40 and hTERT (SH) to a precancerous state and then with H-Ras to a cancerous state (SHR) to analyze genistein effects. Reactivation of the cell-cycle regulators p16 and p21 by genistein was associated with enhanced binding of the activating acetylated histone H3 and reduced binding of the repressive histone markers H3K9me3 and H3K27me3 to their promoter regions.

Table 6.9 Effects mediated by genistein and soy isoflavones on enzymes modifying histones and non-histone proteins.

Mechanism/target	Effect	Cell line/tissue	Genes/factors analyzed	References
Histone acetylation	Induction	*In vitro*	↑ ac-histones, mediated via ER activation and recruitment of coactivator HATs	Hong *et al.* (2004)
	Reduction	MCF7 after long-term genistein exposure	↓ H3 expression and acetylation response after HDACi treatment ↓ growth rate, ↓ response to mitogens (EGF), TSA, and apicidin	Jawaid *et al.* (2010)
	Induction	MDA-MB-231 *in vitro* and as xenografts, C3(1) SV40 TAg mice	↑ acH3, H3K9ac, acH4 at the ER-α promoter, especially in combination with TSA ↓ cell growth, ↓ growth of MDA-MB-231 xenografts and mammary tumors in c3(1) mice	Li *et al.* (2013b)
	Induction	HMEC transformed with SV40, hTERT (SH) and H-Ras (SHR) *in vitro* and in xenografts	↑ acH3, ↓ H3K27me3, H3K9me3 at p21 and p16 promoter expression ↑ p21, p16, ↓ Bmi-1, c-Myc ↓ xenograft growth	Li *et al.* (2013a)
	Induction	SW480, HCT15	↑ acH3 at the DKK1 promoter, ↓ cell growth	Wang *et al.* (2012)
HDAC6 expression	Reduction	LNCaP	↑ acetylation of HSP90, promoting dissociation and degradation of AR	Basak *et al.* (2008)
SIRT1 activity/expression	Reduction	LNCaP, PC-3	↑ ac-H3K9, ↓ H3K9me2, ↓ Akt signaling through PTEN, CYLD, p53, FOXO3a	Kikuno *et al.* (2008)
HAT expression: *p300, PCAF, CREBBP, HAT1*	Induction	LNCaP, DuPro	↑ ac-H3, ac-H4, ↑ p21, $p16^{INK4a}$ expression	Majid *et al.* (2008)
Histone methylation: H3K27me3	Reduction	Eker rats, uterine tissue	Mediated via Akt activation and ↑EZH2 phosphorylation, ↑ hypersensitivity of ER-responsive genes ↑ uterine tumor incidence and multiplicity	Greathouse *et al.* (2012)

In a xenograft model with the SHR cell line, xenograft growth was strongly inhibited by dietary genistein (250 mg/kg).

Dickkopf 1 (DKK1) is an important regulator of Wnt signaling, but it is often lost during colon carcinogenesis (e.g. by DNA methylation). Wang *et al*. (2012) demonstrated that genistein treatment of colon-cancer cell lines leads to reactivation of DKK1 expression and inhibition of cell growth. Induction of DKK1 expression was less pronounced in colon-cancer cell lines with a highly methylated DKK1 promoter, and genistein treatment did not reduce methylation levels. Rather, in cell lines with unmethylated DKK1, such as SW480 and HCT15, genistein treatment led to enhanced binding of acetylated H3 to the DKK1 promoter and coding region. Induction of DKK1 following TSA treatment confirmed that enhanced histone acetylation was causally linked to DKK1 re-expression (Wang *et al.*, 2012).

Several studies have also investigated whether epigenetic mechanisms might be involved in the prostate-cancer chemopreventive activity of genistein. AR signaling and androgen-mediated DNA damage is strongly involved in prostate carcinogenesis (Weischenfeldt *et al.*, 2013). Under basal conditions, AR is stabilized by interaction with the protein chaperone heat-shock protein 90 (HSP90). Basak *et al.* (2008) observed a dose-dependent reduction of AR protein levels in genistein-treated LNCaP prostate-cancer cells. AR mRNA was only reduced at the highest concentration of genistein (50 µM). Instead, genistein treatment prevented the AR-HSP90 interaction via enhanced acetylation of HSP90 and increased ubiquitination and subsequent degradation of the AR. Mechanistically, the anti-estrogenic activity of genistein led to down-regulation of HDAC6, which is important for HSP90 deacetylation. The genistein-mediated effects on AR expression were mimicked by knock down of HDAC6. These data demonstrate that the prostate cancer-preventive potential of genistein may be mediated by an indirect anti-androgen mechanism (Basak *et al.*, 2008).

In prostate cancer, AKT signaling is often aberrantly activated through loss of the tumor-suppressor gene (TSG) PTEN. Other TSGs downstream of PTEN, such as p53, the deubiquitination enzyme CYLD (cylindromatosis), and FoxA3, are often also lost by mechanisms independent of DNA methylation. Kikuno *et al.* (2008) showed that genistein treatment of LNCaP and PC-3 prostate-cancer cell lines reactivated PTEN expression, inactivated AKT, and induced the downstream effectors p53 and FOXO3a. Genistein treatment also led to the up-regulation of CYLD and inhibition of NF-κB. These effects were linked to demethylation (PTEN and CYLD) and increased acetylation (PTEN, CYLD, p53, FOXO3a) of histone H3K9. The authors could demonstrate that H3K9 hyperacetylation was mediated by the down-regulation and reduced nuclear localization of SIRT1, a class III HDAC, highlighting the importance of epigenetic mechanisms for the regulation of AKT and NF-κB signaling by genistein (Kikuno *et al.*, 2008). In another study with LNCaP prostate-cancer cells, genistein treatment led to up-regulation of HATs including p300, PCAF, CBP, and HAT1. As a consequence, acetylation of the histones H3 and H4 increased and the cell-cycle inhibitors p21 and p16 were up-regulated by enhanced association of acetylated H3K4 with their promoters (Majid *et al.*, 2008).

Information on the influence of natural products on histone methylation is still scant. In a study to determine whether exposure to genistein and the estrogenic plasticizer bisphenol A

(BPA) would increase the risk for uterine tumorigenesis, Greathouse *et al*. (2012) identified the HMT EZH2 as a target of genistein-induced PI3K/AKT-mediated nongenomic ER signaling. For developmental reprogramming analyses, Eker rats were postnatally exposed to BPA (50 mg/kg b.w.), estradiol (1 mg/kg), or genistein (50 mg/kg) by subcutaneous injection on days 10–12, and sacrificed at 3 month of age. For acute exposure studies, Eker rats were sacrificed 6 hours after a single injection to the estrogenic compounds on postnatal day 12. Exposure to genistein, but not BPA, led to uterine activation of AKT, enhanced phosphorylation of EZH2, reduced levels of the H3K27me3 repressive methyl marker, and consequently reactivated gene expression. Developmental reprogramming by genistein also promoted uterine tumorigenesis in adult Eker rats. These data indicate that differential epigenetic effects mediated by nongenomic ER signaling can contribute to differences in developmental reprogramming and tumorigenic response to environmental estrogens such as genistein and BPA.

6.5.2.3 miRNAs affected by isoflavones

Genistein and soy isoflavones have been reported to modulate gene expression via a miRNA-related mechanism (Table 6.10). Pancreatic cancer is a cancer type with very poor prognosis, due to late detection and development of drug resistance. EMT is a phenomenon observed during development of drug resistance and cancer-cell metastasis and has been related to altered miRNA expression, such as down-regulation of members of the miR-200 and let-7 families (Li *et al.*, 2009b). Li *et al.* (2009b) addressed the question of whether an isoflavone mixture (containing 70.5% genistein, 26.3% daidzein, and 0.3% glycitein) might affect EMT in gemcitabine-resistant pancreatic-cancer cells. miRNA profiling identified 67 out of 711 miRNAs as differentially expressed after treatment with 25 μM isoflavone mixture (calculated as genistein) for 48 hours. Up-regulation of members of the let-7 (let-7b, let-7e) and miR-200 families (miR-200b, miR-200c) was associated with a change in morphology to a more epithelial-like phenotype and confirmed at the miRNA level by quantitative RT-PCR. After treatment of pancreatic cancer cells with low-dose isoflavones (10 μM) for 3 weeks, the epithelial marker E-cadherin was up-regulated at the mRNA and protein level, whereas the mesenchymal markers ZEB1, vimentin, and slug were down-regulated (Li *et al.*, 2009b), indicating isoflavone-induced reversal of the EMT phenotype. Similar effects were observed in FoxM1 and Notch-1 over-expressing cell lines also characterized by an EMT phenotype (Bao *et al.*, 2011a,b).

Pancreatic cancer cells are characterized by over-expression of EGFR (epidermal growth factor receptor) and activation of the NF-κB signaling pathway. Li *et al.* (2010b) related these observations to lower expression of miR-146 in pancreatic cancer cells than in normal pancreatic-duct epithelial cells. Up-regulation of miR-146 reduced expression of EGFR as well as NF-κB, IκB, IRAK-1 (interleukin 1 receptor-associated kinase 1), and metastasis-associated protein 2 (MTA-2) and reduced the invasive capacity of the cells. Interestingly, all these effects could be mimicked by up-regulation of miR-146 after incubation with 25 μM isoflavones (Li *et al.*, 2010b).

Minichromosome maintenance (MCM) genes play a role in DNA replication during cell division and are often up-regulated in cancer cells, including prostate cancer. Conversely, miR-1296, which has been shown to regulate the expression of MCM2, is down-regulated

Table 6.10 Effects mediated by genistein and soy isoflavones on miRNA expression (verified by RT-PCR).

Mechanism/target	Effect	Cell line/tissue	Genes/factors analyzed	References
let-7b, let-7e, miR-200b, miR-200c	Up-regulation	MiaPaCa-2, L3.6pl, Panc-1	↑ E-cadherin as epithelial marker ↓ vimentin, Slug, Zeb1, expression ↓ reversal of EMT	Li *et al.* (2009b), Bao *et al.* (2011a,b)
miR-146	Up-regulation	Colo357, Panc-1	↓ expression of EGFR, IRAK-1, NF-κB, MTA-2 ↓ invasive capacity	Li *et al.* (2010b)
miR-1296	Up-regulation	LNCaP, PC3	↓ MCM genes and CDK2, CDK7, CDT1	Majid *et al.* (2010a)
miR-29a, miR-1256	Up-regulation	C4-2B, LNCaP	↓ TRIM68, PGK-1 mRNA expression ↓ cell growth and invasion	Li *et al.* (2012)
miR-221, miR-222	Reduction	PC3	↑ ARHI expression (↓ cell proliferation, colony formation, invasion)	Chen *et al.* (2011)
miR-27a	Reduction	C918 *in vitro* and *in vivo*	↑ ZBTB10 expression ↓ xenograft growth (i.p. application 25–100 mg/kg/day)	Sun *et al.* (2009)
miR-21	Reduction	A-498 *in vitro* and *in vivo*	↓ xenograft growth (pretreatment with 25 µM genistein for 4 days)	Zaman *et al.* (2012)

in prostate tumor tissue compared with samples of benign prostatic hyperplasia (BPH) (Majid *et al.*, 2010a). Over-expression of miR-1296 reduced MCM2 mRNA expression and induced cell-cycle arrest in S phase. Similarly, treatment of prostate-cancer cell lines with genistein concentrations of 25 and 50 µM induced miR-1296 up to fivefold. Therefore, the antiproliferative activity of genistein may involve a miRNA-mediated effect on cell-cycle progression (Majid *et al.*, 2010a). Li *et al*. (2012) recently observed the low expression of miR-1256 and miR-29 in prostate-cancer tissue and cell lines and associated it with partial promoter methylation. They identified TRIM68 (tripartite motif containing 68; involved in AR-mediated transactivation) and phosphoglycerate kinase 2 (PGK-1; has important functions in glycolysis), both often up-regulated in prostate cancer, as novel targets of miR1256 and miR-29a. The treatment of prostate-cancer cell lines with 20 µM isoflavone mixture for 5 days reduced the methylation of both miRNA promoters and led to re-expression of both target genes. This was associated with reduced cell growth and invasion. On the other hand, isoflavone treatment reduced the expression of oncomiRs miR-155 and miR-421 (Li *et al.*, 2012).

Chen *et al*. (2011) also investigated the activity of genistein on miRNA expression in prostate cancer. ARHI (age-related hearing impairment 1) is an imprinted tumor-suppressor gene that is down-regulated in prostate cancer. Over-expression studies confirmed its role in the regulation of cell proliferation, colony formation, invasion, and induction of apoptosis (Chen *et al.*, 2011). ARHI was identified as a novel target of the miRNAs miR-221 and -222, which are both up-regulated in prostate cancer. In PC3 prostate-cancer cells treated with 50 µM genistein for 4 days, genistein up-regulated ARHI levels by regulating miR-221 and -222 (Chen *et al.*, 2011).

In another study, Sun *et al.* (2009) demonstrated that genistein treatment at concentrations of 10–200 µM dose-dependently reduced proliferation of uveal melanoma cells *in vitro* and in a xenograft model when applied intraperitoneally at a dose of 25, 50, and 100 mg/kg b.w./day for 1 month. They additionally investigated the effect of genistein on expression of miR-27a, which is often over-expressed in cancer cells, and its target gene ZBTB10 (zinc finger and BTB domain containing 10). In the uveal melanoma cell line, genistein at the highest dose reduced miR-27a levels by about 50% and led to about a 1.3-fold increased expression of ZBTB10 (Sun *et al.*, 2009). The effect on miR-27a and ZBTB10 expression was not analyzed in the xenograft model. Since high doses of genistein were used in this study, and reversal of the effects by transfection of miR-27a was not performed, the observed inhibition of cell growth might be caused by alternative mechanisms unrelated to the modulation of miR-27a expression.

MiR-21 is an oncomiR that is frequently over-expressed in various types of cancer (Zaman *et al.*, 2012). Zaman *et al.* (2012) were interested in determining miR-21 expression and the effect of genistein intervention in renal-cell carcinoma. In this study, kidney-cancer patients with high miR-21 expression had a significantly lower 5-year survival than patients with low miR-21 expression. MiR-21 was shown to be involved in the regulation of apoptosis, migration, and invasion, and regulated expression of the cell-cycle inhibitor p21. Treatment of A-498 renal cancer cells with 25 µM genistein reduced miR-21 expression by about 30%. Pretreatment of A-498 cells with genistein also reduced tumor growth in a xenograft model. Under these conditions, miR-21 expression was about 40% lower than in control tumors.

So far it is not clear how genistein treatment influences miRNA expression. Demethylation of miRNAs silenced by promoter methylation might be relevant to the re-expression of tumor-suppressor miRNAs. Further studies are needed to explore how natural products such as genistein might lead to down-regulation of oncomiRs.

6.6 Conclusions

This chapter has summarized current knowledge of the potential of chemopreventive polyphenols to affect several epigenetic mechanisms, focusing on DNA methylation and histone modifications, including acetylation and methylation. The effects of miRNAs on gene expression were not broadly covered, with the exception of EGCG and genistein.

6.6.1 DNA methylation

Multiple polyphenols of various structural classes have been shown to inhibit DNMT enzyme activity *in vitro*, with more limited and less consistent reports on demethylating activity in cultured cell lines. At present, there is no conclusive understanding as to why some of the data reported by one group are not easily reproducible by another. One reason might be the methodology chosen to detect DNA methylation levels. In earlier studies, proof of demethylating activity in cell culture was often obtained by methylation-specific PCR (MSP), based on the design of selective primer pairs for exclusive amplification of methylated or unmethylated DNA sequences. This method was initially developed for the ultra-sensitive detection of methylated DNA as a cancer biomarker in a pool of unmethylated DNA (Herman *et al.*, 1996), but it might not be suitable for the quantitative detection of a reduction in DNA methylation, as is expected when treating cancer cells with demethylating agents. More reliable state-of-the-art methodology for the quantification of sequence-specific DNA methylation levels includes bisulfite sequencing, pyrosequencing, and the mass spectrometry-based Sequenom MassArray technology (for a summary of these methods, see supplement in Huang *et al.*, 2011). These tools for the sequence-specific quantification of DNA methylation should be preferentially used to quantify demethylating activity.

With the exception of EGCG and genistein, among a few others, the effects of polyphenols on DNA methylation *in vivo* are still limited, and a conclusive link between modulation of DNA methylation levels and cancer preventive efficacy is often still missing. Also, most studies have focused on only a few selected candidate genes, and effects on genome-wide DNA methylation are largely unknown. With the recent advancement of array- and sequencing-based methods for the assessment DNA methylation at a genome-wide scale (Rizzo & Buck, 2012), these analyses are now feasible and affordable, and should be included in future chemoprevention studies in animal models and pilot studies in humans.

6.6.2 Histone-modifying enzymes

The development of HDAC inhibitors has been a focus of medicinal chemistry and the pharmaceutical industry for more than 2 decades. Structural requirements for the inhibition of HDACs are well defined. So far, no flavonoid-type HDAC inhibitor has been identified,

most likely due to the spatial restriction of the active binding pocket of HDACs. In contrast, several sulfur-containing chemopreventive agents, such as metabolites of isothiocyanates from cruciferous vegetables, have been identified as potent HDAC inhibitors *in vitro* and *in vivo*. Some polyphenolic compounds, such as curcumin, EGCG, and genistein, however, have been reported to modulate HDAC activity, histone acetylation, and chromatin accessibility by affecting the expression of HDAC isoenzymes. Interestingly, various polyphenols have been identified as inhibitors of histone acetyl transferases (HATs). This might seem contradictory to the development of HDAC inhibitors for cancer treatment and prevention. However, HAT inhibition should lead to reduced histone acetylation and more condensed chromatin, making it less susceptible to DNA-damaging agents. Furthermore, HATs acetylate not only histones but also a series of important signaling molecules, such as p53, the NF-κB subunit p65, and the AR. Since both NF-κB and AR signaling become deregulated during carcinogenesis, HAT inhibition may indirectly contribute to the anti-inflammatory and anti-androgenic activities of some chemopreventive polyphenols.

6.6.3 miRNAs

Analysis of the influence of miRNAs and other noncoding RNAs on gene regulation is an emerging topic in current epigenetic research. Numerous recent reports based on microarray detection of miRNAs have described multiple changes in miRNA levels following treatment of cancer-cell lines with chemopreventive polyphenols. Interpretation of the data is often hampered by the fact that information on the validated gene targets of miRNAs is limited. Also, most studies do not include mechanistic insights into how chemopreventive polyphenols modulate the expression of miRNAs. These questions should be addressed in future studies.

6.6.4 Summary

In conclusion, 10 years of research have provided a wealth of information on how polyphenols and other chemopreventive agents influence key epigenetic mechanisms mainly in *in vitro* systems. Epigenetic research is highly dynamic and rapidly evolving, both in terms of characterization of epigenetic enzymes in basic research and as drug targets and in terms of technological developments in the detection of DNA methylation and chromatin modifications. Future studies are challenged to make use of these novel technologies in order to translate the *in vitro* findings of chemopreventive polyphenols on epigenetic mechanisms to the *in vivo* situation and to demonstrate the significance of epigenetic gene regulation for chemopreventive efficacy.

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