Chapter 18
Combination Cancer Chemoprevention by Targeting the Epigenome

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Abstract The past 15 years have provided a wealth of information on the influence of natural products and dietary agents on epigenetic mechanisms, including DNA methylation, histone acetylation and methylation, and miRNAs. This chapter will give an overview of studies which have investigated potential additive or synergistic effects of chemopreventive agents targeting the epigenome when used in combination. These studies have focused mainly on breast and colon cancer and investigated green tea catechins and soy isoflavones, quercetin, resveratrol and pterostilbene, withaferin A, the short chain fatty acid butyrate, sulforaphane, selenium, curcumin, synthetic triterpenoids, and docosahexaenoic acid (DHA). Up to now, investigations were limited to in vitro cell culture and animal models. The most promising finding might be the reactivation of the estrogen receptor in estrogen receptor-negative breast cancer by various combinations of DNA demethylating and histone-modifying compounds, increasing susceptibility to anti-hormonal therapy.

18.1 Introduction

The term "epigenetics" refers to modifications in gene expression caused by heritable, but potentially reversible, changes in DNA methylation and chromatin structure, without alterations in DNA sequence (Felsenfeld 2014). Major epigenetic mechanisms include DNA hyper- and hypomethylation (Jones 2012), remodeling of the chromatin, modification of histones by histone acetylation and methylation (among others) (Barnes et al. 2019), and non-coding RNAs (Guil and Esteller 2009).

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18.1.1 DNA Methylation

Methylation at the C5 position of cytosines in the context of CpG dinucleotides is the most prevalent DNA-based epigenetic mark in the human genome. Considering that all cells of an organism share the same genomic information, an important feature of DNA methylation is that it regulates gene transcription in a cell-type specific manner. The DNA methyltransferase (DNMT) family of enzymes catalyzes the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to DNA and maintains DNA methylation patterns after DNA replication. DNMT1, the maintenance methyltransferase, preferentially methylates hemimethylated DNA, whereas the methyltransferases DNMT3a and 3b are involved in methylation of fully unmethylated DNA. With few exceptions related to cell type-specific gene expression patterns, in healthy tissue, CpG-dense regions (so-called CpG islands, CGIs), located in the promoter regions of about 60% of all genes, are usually unmethylated. On the other hand, intra- and intergenic regions with lower CpG density are usually highly methylated, thus limiting accessibility of DNA and maintaining genomic stability. Also, repetitive genomic sequences are highly methylated to prevent these sites from active transcription (Stirzaker et al. 2014). Methylation at enhancer regions may protect from transcription factor binding, thus fine-tuning gene transcription. Inhibition of DNMT1 activity or downregulation of DNMT1 expression leads to passive loss of DNA methylation during cell division. Alternatively, ten-eleven translocation (TET) proteins are involved in active demethylation and can reactivate previously silenced genes (Feinberg et al. 2016).

During carcinogenesis, focal gain in methylation at CGIs in promoter regions, for example of tumor suppressor genes (TSGs), concomitant with global loss of methylation (hypomethylation), especially at repetitive sequences, is thought to be involved in the etiology of cancer (Esteller 2007; Berdasco and Esteller 2010). In contrast to irreversible genetic alterations (by mutations, deletions etc.), alterations in gene expression via epigenetic mechanisms are reversible. Consequently, aberrant methylation has been identified as an attractive target for cancer chemoprevention with dietary compounds.

18.1.2 Histone Modifications

Chromatin accessibility and gene expression is dynamically controlled by various post-translational modifications of N-terminal histone tails, including acetylation and methylation (Barnes et al. 2019; Kouzarides 2007; Soshnev et al. 2016). Histone acetylation of histone tails is catalyzed by histone acetyltransferases (HATs) that transfer acetyl groups from acetyl-CoA mainly to lysine residues, resulting in opening of the chromatin structure and facilitating transcription factor binding to promoter or enhancer regions to regulate transcription (Voss and Thomas 2018). Histone acetylation is reversed by histone deacetylases (HDACs) that remove

histone acetyl groups, leading to chromatin condensation and transcriptional repression (Minucci and Pelicci 2006). Importantly, the catalytic activity of HATs and HDACs is not limited to histones, and numerous non-histone proteins, including cytoskeletal proteins, molecular chaperones, hormone receptors, nuclear import factors, and transcription factors such as p53 and NF-κB, have been identified as targets (Glozak et al. 2005; Kim et al. 2016). Besides the currently known HDACs 1-11, structurally unrelated sirtuins (SIRTs), which use NAD^+ as a cofactor, possess deacetylating activity (Seto and Yoshida 2014).

In contrast to histone acetylation, histone methylation can have activating or repressive effects on gene expression, dependent on which lysine residue is modified by methylation and how many methyl groups are transferred (Kouzarides 2007). Histone methylation by transfer of methyl groups from SAM to lysine residues is catalyzed by more than 30 histone methyltransferases (HMT) (Upadhyay and Cheng 2011; Allis et al. 2007) and removed by histone lysine demethylases. Again, these enzymes possess non-histone targets including p53, RB1 and STAT3 with important roles in carcinogenesis (Hamamoto et al. 2015). So far, the impact of chemical or natural product inhibitors of histone-modifying enzymes on non-histone proteins is likely largely underestimated in cancer prevention (Kim et al. 2016; Shortt et al. 2017).

18.1.3 Regulation of Gene Expression by Noncoding (Micro) RNAs

MicroRNAs (miRNAs or miRs) are short single stranded RNA oligonucleotides with a length of 20–23 nucleotides. They influence mRNA levels and translation by interacting with a target sequence often in the 3′ untranslated region of a gene, either by perfect base-pairing, leading to mRNA degradation, or by partial base-pairing that blocks translation. Each miRNA is estimated to control several hundred genes involved in key biological processes, including development, differentiation, apoptosis and proliferation (Calin and Croce 2006). The miRBase database currently lists about 1900 human, 1200 mouse, and 500 rat miRs (Kozomara et al. 2019). Biogenesis of miRNAs from RNA precursor structures is highly regulated and involves multiple steps [reviewed in Winter et al. (2009)]. Expression of many miRs is deregulated during cancer development. miRNAs have either tumor suppressive function, such as the miR-200 family (Park et al. 2008), or oncogenic functions (onco-miRs), such as miR-21 upregulated in many types of cancer (Wu et al. 2015). Major mechanisms contributing to their deregulation include genetic and epigenetic alterations such as DNA methylation, as well as defects in the miRNA processing machinery (Brait and Sidransky 2011).

18.2 Combination Effects Targeting the Epigenome

During the past 15 years, the influence of natural products and dietary chemopreventive agents on epigenetic mechanisms has gained major interest in the research community. Only recently, studies are emerging that are based on the combination of chemopreventive agents targeting the epigenome. Already about 40 years ago, Michael Sporn reported about the concept of 'combination chemoprevention of cancer' by combining two or more chemopreventive agents with complementary mechanisms of action to enhance efficacy while reducing toxicity (Sporn 1980). This concept has been taken up in a recent report on cancer chemoprevention by the Division of Cancer Prevention of the National Cancer Institute (Mohammed et al. 2019).

Agents investigated in combination studies with the aim to target two or more epigenetic mechanisms include green tea catechins and soy isoflavones, quercetin, resveratrol and pterostilbene, withaferin A (WA), the short chain fatty acid butyrate, sulforaphane (SFN), selenium, curcumin, synthetic triterpenoids, and docosahexaenoic acid (DHA) (Fig. 18.1).

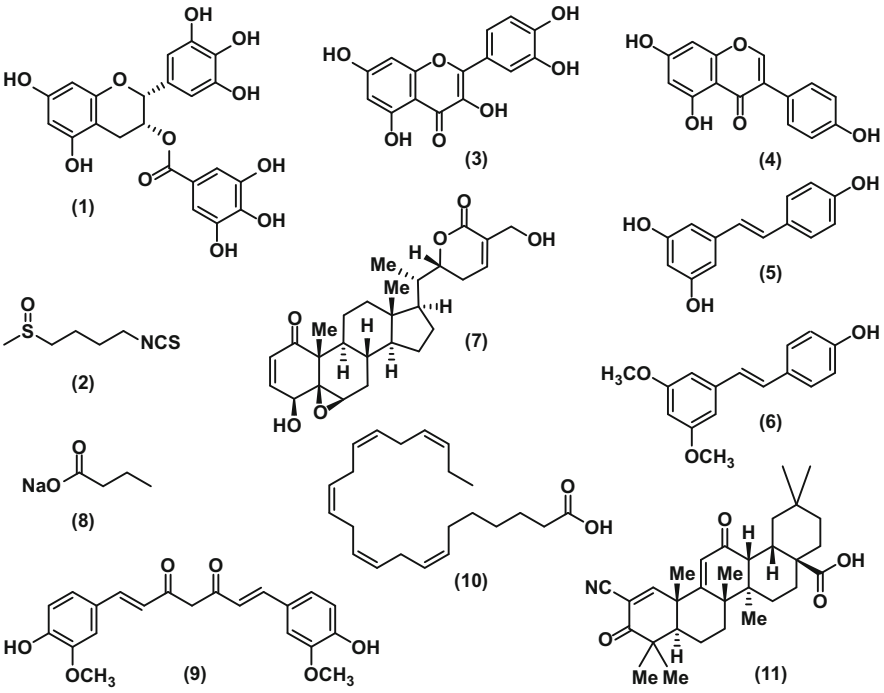

Fig. 18.1 Structures of chemopreventive agents tested in combination studies targeting epigenetic mechanisms. (1) Epigallocatechin gallate (EGCG), (2) sulforaphane (SFN), (3) quercetin, (4) genistein, (5) resveratrol, (6) piceatannol, (7) withaferin A (WA), (8) butyrate, (9) curcumin, (10) docosahexaenoic acid (DHA), (11) 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid (CDDO)

These compounds have been tested in various combinations to explore potential additive or synergistic effects on epigenetic mechanisms, with a focus on breast and colon cancer, as outlined below. Before describing their combination effects, the compounds and their sources are briefly introduced, including a summary of their chemopreventive and epigenetic activities. For a general overview of effects of bioactive compounds on the epigenome, readers are referred to a number of recent review articles on nutri-epigenetics and cancer prevention (Link et al. 2010; vel Szic et al. 2010; Huang et al. 2011, 2019; Vanden Berghe 2012; Gerhauser 2013, 2014, 2018; Shukla et al. 2014; Aggarwal et al. 2015; Shankar et al. 2016; Carlos-Reyes et al. 2019; Gao and Tollefsbol 2015; Biersack 2016).

18.2.1 Breast Cancer Studies

With an estimated number of 2.09 million new cases and about 627,000 cancer deaths in 2018, breast cancer is worldwide the most common cancer in females (Bray et al. 2018). Based on characteristic gene expression patterns, breast cancer is clinically sub-grouped into at least five distinct subtypes (Sorlie et al. 2003). Luminal A/B breast cancers express the estrogen receptor (ER) and genes responsive to ER-signaling. HER2 tumors are characterized by amplification of the *ERBB2* gene, and patients respond to monoclonal antibody therapy with trastuzumab. Basal breast tumors express breast basal cell keratins 5/6 and 17 and overlap with the 'triple negative' breast cancer subtype (TNBC) that does not express ER, progesterone receptor (PR) and HER2, whereas gene expression patterns of the normal-like subtype resemble that of normal breast tissue (Sorlie et al. 2003; Perou et al. 2000). Anti-estrogenic therapies are used to treat luminal breast cancers, but they fail in ER-negative tumors. Epigenetic analyses have indicated that the ERα is epigenetically silenced by promoter methylation [reviewed in Hervouet et al. (2013)]. Combined in vitro treatment of the basal breast cancer cell line MDA-MB-231 with the DNMT inhibitor 5-aza-2′-deoxy-cytidine (decitabine, DAC) and the HDAC inhibitor trichostatin A (TSA) resulted in synergistic ~400-fold elevated ERα mRNA expression, indicating that chromatin de-condensation in combination with DNA demethylation was effective in ERα de-repression (Yang et al. 2001).

A number of studies have evaluated the combined effects of chemopreventive agents in in vitro and in vivo breast cancer models, especially combinations with green tea catechins or sulforaphane (SFN) (Table 18.1).

18.2.1.1 Combination of Green Tea Catechins with Broccoli Sprouts or Sulforaphane (SFN)

Green tea polyphenols (GTP) represent a mixture of compounds, including (−)-epigallocatechin 3-gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC). GTP and especially EGCG have a broad spectrum of

Table 18.1 Combination effects in breast cancer models in vitro and in vivo

Model and optimal treatment	Effects	References
In vitro		
MDA-MB-231, MDA-MB-453, MCF10A Green tea polyphenols (GTP) 20 μg/ml SFN 5 μM	⇧ synergistic reactivation of ERα expression in ER-negative breast cancer cell lines ⇧ activating histone marks to ERα promoter ⇩ repressive histone marks ⇩ DNA methylation at ERα promoter ⇧ sensitivity to tamoxifen (TAM) treatment	Meeran et al. (2012)
MDA-MB-231, MDA-MB-157 EGCG 20 μM SFN 10 μM	⇧ synergistic reactivation of ERα expression ⇩ HDAC1, DNMT1 expression	Li et al. (2017)
HMEC, SH and SHR breast cancer cells EGCG 20 μM SFN 10 μM	⇧ apoptosis, cell cycle arrest in S-phase ⇩ HDAC1, DNMT1 expression ⇧ H3 hyperacetylation ⇧ DNA hyper- and hypomethylation ⇧ DCBLD2 mRNA levels ⇩ Septin 9 mRNA	Li et al. (2016)
MDA-MB-231, MCF7, MCF-10A SFN 5 μM Withaferin A 1 μM	⇩ synergistic reduction of cell growth in MCF7 cells ⇧ induction of apoptosis ⇩ DNMT3A/B protein (MCF7) or mRNA expression (MDA-MB-231) ⇩ HDAC1 mRNA (MCF7) or protein expression (MDA-MB-231) ⇧ upregulation of pro-apoptotic BAX and downregulation of anti-apoptotic BCL2 in both cell lines	Royston et al. (2017)
MDA-MB-231, MCF7 SFN 5 μM Withaferin A 1 μM	⇧ induction of cell cycle arrest ⇩ reduction of Cyclin D1, CDK4, HDAC2 and 3 expression, RB phosphorylation, HDAC2 and 3 ⇧ induction of E2F and p21 expression ⇩ HMT activity ⇧ HAT activity	Royston et al. (2018)
MDA-MB-231, MCF7 SFN 5 μM Genistein 10, 15 μM	⇩ synergistic decrease in cell viability ⇧ synergistic induction of apoptosis ⇧ synergistic induction of G1 (MCF7) or G_2/M (MDA-MB-231) cell cycle arrest ⇩ synergistic reduction of HDAC and HMT activity ⇩ HDAC2 and HDAC3 mRNA and protein expression	Paul et al. (2018)

(continued)

Table 18.1 (continued)

Model and optimal treatment	Effects	References
MDA-MB-231, MDA-MB-157 Genistein 25 μM TSA 100 ng/ml	⇧ synergistic reactivation of ERα expression in ER-negative breast cancer cell lines ⇧ chemosensitivity to tamoxifen treatment Modulation of histone modifications at ERα promoter ⇩ HDAC activity	Li et al. (2013)
HCC1806, MDA-MB-157, MCF10A Resveratrol 15 μM Pterostilbene 5 μM	⇩ colony formation (MDA-MB-157) ⇧ apoptosis ⇧ G_2/M arrest (HCC1806), S-phase arrest (MDA-MB-157) ⇩ SIRT1 protein expression and activity ⇩ DNMT mRNA levels and DNMT activity ⇩ γH2AX foci as a measure of DNA damage and repair (DDR) response ⇩ hTERT mRNA levels and telomerase activity	Kala et al. (2015)
HCC1806, MDA-MB-157, MCF7 Resveratrol 15 μM Pterostilbene 5 μM	⇧ synergistic reactivation of ERα expression ⇧ acH3, acH4, H3K9ac at ERα promoter ⇩ DNMT activity and global DNA methylation (MDA-MB-157) ⇧ sensitivity to 4-hydroxytamoxifen (4-OHT) treatment Modulation of PGR mRNA expression in response to estradiol (E2) or 4-OHT treatment	Kala and Tollefsbol (2016)
Tumorospheres from MMTV-neu-Tg tumors Human breast cancer cell lines MCF10A4, CAL51, murine mammary tumor cell line 4 T1 3D cultures of MMTV-neu-Tg CSCs Butyrate 1 mM 5-Azacytidine 1 μg/ml	⇩ cancer stem cell (CSC) abundance in tumorospheres ⇩ colony formation Differential expression of genes involved in cell cycle regulation, cell division, kinetochore formation, chromosome segregation and mitosis	Pathania et al. (2016)
Raw264.7 murine macrophages, primary macrophages from PyMT mice CDDO-Ea 1, 3, 10 nM or CDDO-Me 1, 3, 10 nM SAHA 100, 300, 1000 nM	⇩ enhanced reduction of INF-γ- or LPS-induced inflammatory response (measured as nitrite) by combination relative to single compounds	Tran et al. (2013)

(continued)

Table 18.1 (continued)

Model and optimal treatment	Effects	References
In vivo		
Nu/nu mice, orthotopic injection of MDA-MB-231 cells into mammary fat pads Green tea polyphenols (0.3% in drinking water) Broccoli sprouts BSp (13% BSp seeds in AIN-93G diet)	⇧ inhibition of tumor growth by combination treatment ⇧ chemosensitivity to tamoxifen treatment ⇧ synergistic reactivation of ERα expression ⇩ HDAC1, DNMT1 expression ⇧ H3 hyperacetylation Modulation of histone marks at ERα promoter ⇧ binding of p300 at ERα promoter ⇩ binding of repressor at ERα promoter	Li et al. (2017)
Nu/nu mice, orthotopic injection of SHR cells into mammary fat pads Green tea polyphenols (0.5% in drinking water) Broccoli sprouts BSp (26% BSp seeds in AIN-93G diet)	Up to 94% inhibition of tumor growth by combination treatment	Li et al. (2016)
C3(1) SV40 TAg mouse model BSp (13% BSp seeds in AIN-93G diet) Genistein (250 mg/kg diet)	⇧ tumor latency ⇩ tumor and volume	Paul et al. (2018)
Nu/nu mice, orthotopic injection of MDA-MB-231 cells into mammary fat pads Genistein (250 mg/kg diet) alone and in combination with TAM (25 mg/pellet)	⇩ tumor growth by genistein alone and especially in combination with TAM, no effect of TAM alone ⇩ tumor weight ⇧ ERα mRNA and protein expression, especially in combination with TAM ⇩ DNMT1, HDAC1 mRNA and protein expression in combination with TAM ⇩ HDAC1 mRNA levels, genistein and TAM alone and in combination	Li et al. (2013)
C3(1) SV40 TAg mouse model Genistein (250 mg/kg diet) alone and in combination with TAM (25 mg/pellet)	⇧ tumor latency ⇧ sensitivity to TAM ⇧ ERα protein expression, alone and especially in combination with TAM ⇩ HDAC1 protein expression, alone and in combination with TAM ⇩ HDAC1 mRNA levels and activity, genistein and TAM alone and in combination ⇩ DNMT1 mRNA levels, genistein and TAM in combination, ⇩ DNMT1 activity, genistein alone and in combination with TAM	Li et al. (2013)

(continued)

Table 18.1 (continued)

Model and optimal treatment	Effects	References
Orthotopic 4 T1 cell injection in Balb/c mice Butyrate (10 mg/21 day release tablets) 5-Azacytidine (0.5 mg/21 day release tablets)	⇧ overall survival	Pathania et al. (2016)
Murine MMTV- polyomavirus middle T (PyMT) model of ER-negative mammary tumors CDDO-Ea 400 mg/kg diet or CDDO-Me 50 mg/kg diet + Suberoylanilide hydroxamic acid (SAHA) 250 mg/kg diet	⇧ efficacy in delaying tumor onset by either triterpenoid in combination with SAHA treatment ⇩ infiltration of tumor associated macrophages (TAM) by CDDO-Me + SAHA ⇩ secreted levels of pro-angiogenic MMP9 ⇩ INF-γ (10 ng/ml) or LPS (3 ng/ml)-induced nitric oxide levels in RAW 264.7 or isolated PyMT macrophages by combination of SAHA (30,100, 300 nM) with CDDO-Me (1,3,10 nM) or CDDO-Ea (3, 10, 30 nM)	Tran et al. (2013)

⇧ induction, enhancement, increase; ⇩ repression, inhibition, reduction; ⇔ no change

chemopreventive activities in vitro. They act as pro- and antioxidants, influence a series of signal-transduction pathways (MAPK, EGFR, NF-κB, IGF), and inhibit cell growth, angiogenesis, and the activity of enzymes relevant for drug metabolism and inflammation (Khan and Mukhtar 2008; Yang et al. 2009; Yang and Wang 2016). Recent research suggests that cancer stem cells are targeted by GTP and EGCG (Fujiki et al. 2018). GTP and EGCG were able to prevent cancer development in animal models of all major organ sites. Although human intervention studies with GTP have provided some promising results (Yang and Wang 2016; Fujiki et al. 2018; Yang et al. 2016), human epidemiological studies were less conclusive. This was attributed mainly to the low quantities of tea consumed (Yang et al. 2009, 2016). GTP have been tested in intervention studies and clinical trials for the prevention and treatment of breast, prostate, colorectal, bladder, lung, skin, esophageal, liver and thyroid cancer (National Library of Medicine 2019).

With respect to epigenetic mechanisms, ECGC and other GTP have been shown to inhibit the activity of DNMTs in vitro and to reduce their expression in cell culture (Carlos-Reyes et al. 2019). As a consequence, EGCG induced reexpression of genes regulating cell cycle progression (p16, p21), cell signaling (*RARβ*), WNT signaling (*WIF-1*), DNA repair (*MGMT, hMLH1*) and apoptosis (*DAPK*). In rodent models and in human epidemiological studies, the influence of EGCG on DNA methylation is inconsistent [review in Gerhauser (2014)]. With respect to posttranslational histone modifications, EGCG was shown to enhance the expression of HATs and to reduce the expression of HDACs, both resulting in increased levels of acetylation

at histone as well as non-histone proteins (e.g., p53). Additionally, EGCG inhibited HAT activity and thereby reduced acetylation of important transcription factors including NF-κB p65 and androgen receptor (AR) [review in Gerhauser (2014) and Gao and Tollefsbol (2015)]. In addition to DNA methylation and histone modifications, EGCG also affected the expression of numerous miRNAs in various cancer cell lines, resulting in cell growth inhibition or induction of apoptosis (Gerhauser 2014; Biersack 2016).

Sulforaphane (SFN) is an isothiocyanate (ITC) found as a precursor glucosinolate in broccoli and other cruciferous vegetables (Verkerk et al. 2009). ITCs are thiol-reactive compounds and act by a broad range of cancer preventive activities, including induction of antioxidant and Phase 2 metabolizing enzymes via the NRF2/KEAP1 pathway (Qin and Hou 2016), induction of cell cycle arrest, apoptosis, and autophagy, as well as anti-inflammatory, anti-proliferative and anti-angiogenic activity (Thomson et al. 2010; Houghton et al. 2013). In addition, ITCs possess antimicrobial properties (Dufour et al. 2015). SFN and other ICTs are currently investigated in clinical trials for breast, prostate, lung and gastrointestinal cancers (National Library of Medicine 2019; Palliyaguru et al. 2018; Tortorella et al. 2015).

About 15 years ago, a cysteine metabolite of SFN was first described to inhibit HDAC activity in vitro (Myzak et al. 2004). This epigenetic effect was also demonstrated in vivo in various tissues and intestinal polyps in the $Apc^{Min/+}$ mouse model. In a small human trial, consumption of fresh broccoli sprouts resulted in rapid and transient inhibition of HDAC activity and histone hyper-acetylation in peripheral blood mononuclear cells (Dashwood and Ho 2007). In various hormone-dependent cancer cell lines SFN downregulated the expression of DNMTs and induced DNA methylation changes, e.g., of the cell cycle regulators cyclin D2 and p21, pro-apoptotic *BAX*, as well as of TSGs *PTEN, RARβ2, CDH1, DAPK1* and *GSTP1*, leading to cell cycle arrest and apoptosis induction [review in Tortorella et al. (2015) and Su et al. (2018)]. In addition, SFN and other ITCs affected the expression of noncoding RNAs, thereby inhibiting cell proliferation, cell migration, invasiveness and epithelial-to-mesenchymal transition (EMT) [reviewed in Martin et al. (2018)].

In 2012, Meeran et al. reported that incubation of ER-negative MDA-MB-231 cells with a green tea polyphenol (GTP) extract (20 μg/ml) in combination with 5 μM SFN for 72 h resulted in ERα reactivation at the mRNA and protein level (Meeran et al. 2012). The co-treatment led to a significant ~40% reduction of DNMT and HDAC activities in nuclear extracts. This was associated with a 40–65% reduction of DNMT1, 3a and 3b protein expression compared to control cells. Also, protein expression of HDAC1, 4, and 6 as well as of H3 lysine 9 methyltransferase SUV39H1 (also known as KMT1A) was strongly reduced. Consequently, global acetylation of histones H3 and H4 as well as the activating mark H3K9ac time-dependently increased at the ERα promoter, whereas the repressive mark H3K9me3 declined. Bisulfite sequencing indicated that GTP + SFN treatment lowered DNA methylation at the ERα core promoter. Chromatin immunoprecipitation (ChIP)-PCR experiments confirmed that the combined treatment significantly reduced binding of

a repressor complex composed of the epigenetic writers DNMT1, HDAC1 and SUV39H1 as well as of the methyl-binding proteins MBD1 and MeCP2 to the ERα promoter. Epigenetic de-repression of ERα re-sensitized MDA-MB-231 cells to treatment with the selective estrogen receptor modulator (SERM) tamoxifen (TAM). This was indicated by significant ER-dependent induction of apoptosis by co-treatment with GTP, SFN and TAM (Meeran et al. 2012).

Similar results were obtained when MDA-MB-231 cells were treated with EGCG (20 μM) in combination with 10 μM SFN (Li et al. 2017). To confirm that the observations were relevant in vivo, Li et al. treated athymic nude mice with GTP (0.3% in drinking water) and broccoli sprouts BSp as a source of SFN (13% BSp in diet) alone and in combination for 2 weeks, and then injected MDA-MB-231 cells orthotopically into mammary fad pads. Both single as well as the combined treatment especially in combination with TAM significantly reduced xenograft growth. Consistent with the in vitro results, the combination treatment with GTP + BSp resulted in re-expression of ERα protein, reduction of HDAC1 and DNMT1 protein expression, elevated levels of H3ac, H4ac, H3K9ac and the histone acetyltransferase p300 at the ERα promoter, whereas SUV39H1 and H3k9me3 levels were reduced (Li et al. 2017). These results suggest that re-sensitizing TNBC cells to anti-hormonal therapy by combined opening of the chromatin and demethylation of the ER promoter is a feasible approach to reduce TNBC growth.

In a previous study, Li et al. had investigated the combination of EGCG and SFN in an in vitro model of early stages of breast cancer cellular transformation (Li et al. 2016). Normal human mammary epithelial cells (HMECs) were transfected with *SV40* and *hTERT* to generate ER-negative early transformed precancerous SH cells, or additionally with *H-Ras* to produce completely transformed breast cancer cells (SHR cells). Combined treatment with 20 μM EGCG and 10 μM SFN reduced cell growth in transformed cells, but not in normal HMECs, and induced apoptosis and cell-cycle arrest in S-phase. In SHR cells, EGCG and SFN alone and in combination reduced HDAC1 and DNMT1 mRNA expression and activity. SFN treatment alone and in combination with EGCG led to H3 hyper-acetylation. Genome-wide methylation changes at 485,000 CpG dinucleotides were assessed using Illumina 450k BeadChip arrays in SHR cells. Both compounds alone and in combination differentially affected DNA methylation at 266 CpG sites with methylation differences larger than 20%. The combination of EGCG and SFN induced more prominent changes (both hyper- and hypomethylation) than the single agents. Affected genes were enriched for chromosomal rearrangement, RNA binding, differentiation and development. In vivo, the combination of GTP (0.5% in drinking water) as a source of EGCG and BSp (26% BSp in the diet) as a source of SFN significantly and additively reduced SHR xenograft growth when the cells were injected into mammary fat pads of nude mice (Li et al. 2016).

Overall, these studies showed that dietary interventions targeting complementary epigenetic mechanisms can re-sensitize ER-negative tumors to anti-hormonal therapy. The results demonstrated that combined application of GTP + BSp in combination with anti-hormonal treatment might be a feasible treatment option for breast cancer patients with ER-negative tumors, which should be further evaluated in clinical trials.

18.2.1.2 Combination of SFN with Withaferin A (WA)

Withania somnifera, also known as Indian ginseng, is used in traditional Ayurvedic medicine to treat diseases with a broad spectrum of indications from diabetes to cancer (Vyas and Singh 2014; Gauttam and Kalia 2013; Palliyaguru et al. 2016). Beside other bioactive compounds (flavonoids, tannins), *Withania somnifera* is rich in steroidal lactones known as withanolides. Withaferin A (WA) bears a reactive α,β-unsaturated carbonyl group, which has been associated with its bioactivities by interaction with cysteine thiol-residues of intracellular signaling molecules (Lee and Choi 2016). WA affects a broad spectrum of chemopreventive mechanisms. Its pro-oxidative activity is linked to induction of apoptosis. WA also targets various signaling pathways, including KEAP1/NRF2 (Heyninck et al. 2016), NF-κB, STAT3, NOTCH, mitogen-activated protein kinase (MAPK), p53, and estrogen receptor signaling. Further, WA was shown to influence stress response by inducing heat shock response and to inhibit angiogenesis. Various studies have reported that WA inhibits carcinogenesis in chemically-induced rodents models for head and neck, mammary gland and skin cancer. It was also shown to suppress growth of prostate, breast, thyroid, cervical, lung and colon cancer cells in cancer xenograft models [reviewed in Vyas and Singh (2014), Palliyaguru et al. (2016), and Lee and Choi (2016)].

With respect to epigenetic mechanisms, WA reduced the expression of DNMTs in both ER-dependent and -independent breast cancer cell lines (Mirza et al. 2013). Conversely, WA treatment led to DNA hypermethylation and downregulation of selected genes related to cancer invasiveness in TNBC cells (Szarc Vel Szic et al. 2017). It was further shown to decreases histone H3 acetylation and transcription factor recruitment to the interleukin IL-6 gene promoter and thus abolished IL-6 gene expression in TNBC cells (Ndlovu et al. 2009).

SFN (5 μM) was tested in vitro in combination with WA (1 μM) in ER-positive (MCF7) and negative (MDA-MB-231) breast cancer cell lines (Royston et al. 2017, 2018). In both cell lines, the combination of SFN + WA reduced cell viability and induced apoptosis. The combination also lowered DNMT activity as well as DNMT1, 3a and 3b and HDAC1 mRNA and protein expression, often more effective than either compound alone. With respect to apoptosis induction, SFN + WA induced protein expression of pro-apoptotic BAX and reduced anti-apoptotic BCL-2 (Royston et al. 2017). The authors could further show that SFN + WA induced G1 cell cycle arrest by modifying the expression of cell cycle regulating genes, including p21. Further, protein expression of HDAC2 and 3 were lowered, histone methyl transferase (HMT) activity was reduced, and HAT activity was induced, especially in MDA-MB-231 cells (Royston et al. 2018).

These results indicate that by combining SFN and WA, cell growth inhibition by induction of cell cycle arrest and apoptosis was achievable at lower concentration than with either compound alone. Since both compounds possess a broad spectrum of bioactivities, the mechanism of inhibition induced by the combination should be further investigated in vitro and in animal models for breast cancer.

18.2.1.3 Combination of SFN with Soy Isoflavones

Soybean (*Glycine max* L.) and soy products contain high levels of isoflavones such as genistein and daidzein with phyto-estrogenic properties (Xiao et al. 2018). A traditional soy-rich, low-fat Asian diet is generally associated with a reduced risk for breast and prostate cancer (Xiao et al. 2018; Messina 2016; Mukund et al. 2017; Russo et al. 2016; Magee and Rowland 2012). Beside estrogen receptor-mediated signaling, genistein and other soy isoflavones act by additional chemopreventive mechanisms, including inactivation of carcinogens and reactive oxygen species as well as inhibition of cell-signaling, inflammation, angiogenesis, cell cycle progression, and induction of apoptosis (Magee and Rowland 2012; Steiner et al. 2008; Molinie and Georgel 2009). In various rodent models for cancer prevention, isoflavones have been shown to prevent major cancer types (Banerjee et al. 2008). Genistein is tested in various clinical trials for treatment and prevention of prostate, bladder, kidney, breast, colorectal, lung, pancreas and endometrial cancer (National Library of Medicine 2019; Taylor et al. 2009).

Isoflavone interaction with ER leads to recruitment of nuclear co-activators or co-repressors, which have histone modifying function and modulate the chromatin structure. Various in vitro and in vivo studies have demonstrated potential of genistein and soy isoflavones to target the activity or expression of enzymes functioning as epigenetic writers, readers and erasers and to influence miRNA expression. These activities affect genes associated with the major hallmarks of cancer, and lead to activation TSGs such as *RARβ2, BTG3, PTEN* and *ATM*, genes involved in DNA repair (*MGMT, BRCA1, BRCA2, GSTP1*), cell signaling (especially Wnt-signaling, e.g., *APC, SOX7, WIF1, DKK1, SFRP1, SFRP2*), epigenetic (*EZH2, SRC3, p300*) and cell cycle regulators (p16, p21), estrogen receptors (ER-α, ER-β) and genes associated with EMT (e.g., *ZEB1/2, VIM*). This is of relevance for all major cancer types [comprehensive review in Gerhauser (2014, 2018) and Pudenz et al. (2014)], although a causative link between the influence on epigenetic mechanisms and cancer prevention is still missing.

Similar to the study of SFN and WA (Royston et al. 2018), the combination of SFN (5 μM) with genistein (10 or 15 μM, respectively) was tested in ER-positive (MCF7) and negative (MDA-MB-231) breast cancer cell lines. SFN + genistein inhibited cell proliferation and induced apoptosis and cell cycle arrest of both cell lines (Paul et al. 2018). The authors reported reduced HDAC and HMT activity in MCF-7 and MDA-MB-231 cells treated with the combination, but not with either compound alone. Normal MCF10A cells were not affected. Also, mRNA and protein expression of HDACs 2 and 3 were most reduced by the combined compounds. In the C(3)1 SV40 TAg transgenic mouse model, combination of genistein (250 mg/kg diet) with BSp (13% in diet) (representing human daily consumption of about 2 g isoflavones and 2 cups of broccoli) most effectively increased tumor latency and reduced average tumor volume by about 50% (Paul et al. 2018).

These results support the enhanced benefit of combining a soy-based diet with cruciferous vegetables such as broccoli sprouts for the prevention of breast cancer development.

18.2.1.4 Combination of Genistein with the HDAC Inhibitor Trichostatin A (TSA)

Li et al. investigated the combination effects of genistein in combination with the HDAC inhibitor trichostatin A (TSA) (Li et al. 2013). TSA is an antifungal antibiotic with cytostatic and differentiating activity and is classified as a pan-HDAC inhibitor (Vigushin et al. 2001; Kim and Bae 2011). In vitro, treatment of MDA-M-231 cells with 25 μM genistein in combination with 100 ng/ml TSA resulted in synergistic ERα re-expression, reduced HDAC activity, reduced HDAC1 and DNMT1 expression and binding to the ERα promoter, as well as in elevated levels of activating histone marks (H3ac, H4ac, H3K9ac) at the ERα promoter.

In vivo, dietary genistein (250 mg/kg diet) fed for 2 weeks prior to orthotropic injection of MDA-MB-231 cells reduced xenograft growth. Importantly, genistein re-sensitized the tumor cells to treatment with TAM (25 mg pellet implanted 2 weeks after xenograft injection). Genistein intervention combined with TAM inhibited xenograft growth by >95%. In the C(3)1 SV40 TAg transgenic mouse model for basal breast cancer, genistein increased tumor latency and response to TAM treatment. Mechanistically, genistein alone and in combination with TAM inhibited tumor cell proliferation measured by PCNA staining and led to re-expression of ERα protein. In both the xenograft and the transgenic mouse model, genistein in combination with TAM significantly reduced DNMT1 and HDAC1 mRNA and protein expression and activity (Li et al. 2013).

This study is another demonstration that the ER can be re-activated in basal or TNBC models by combined treatment with HDAC inhibitors and compounds modulating DNA methylation, resulting in enhanced response to anti-hormonal treatment.

18.2.1.5 Combination of Two Stilbenes Resveratrol and Pterostilbene

Resveratrol and pterostilbene are plant-derived stilbene derivatives found in the skin of red grapes, blueberries and in other fruits (Rimando and Suh 2008). Resveratrol was first described as a cancer chemopreventive agent in 1997 and has a broad spectrum of health-beneficial effects, including anti-oxidant, cardio-protective and anti-tumor activities (Pezzuto 2008). Mechanistically, these activities have been linked to the interaction with hormone receptors, influence on drug metabolism, and anti-inflammatory, anti-proliferative, anti-angiogenic and anti-metastatic properties, as well as pro-apoptotic activity (Rimando and Suh 2008; Pezzuto 2008; Kundu and Surh 2008; Pavan et al. 2016). Resveratrol and pterostilbene have been shown to reduce inflammation and to prevent carcinogenesis in animal models for

colon cancer, as well as some other cancer types (Rimando and Suh 2008). Resveratrol is rapidly metabolized and plasma levels after oral consumption are low (Baur and Sinclair 2006). Bioavailability can be modulated by various factors including formulation, matrix effects, time of consumption and combination with modulators of resveratrol metabolism (Ramirez-Garza et al. 2018). Resveratrol is currently tested in several human intervention trials, with a focus on colon cancer prevention, which does not require systemic uptake (National Library of Medicine 2019; Pezzuto 2008, 2019; Pavan et al. 2016).

In earlier reports, resveratrol had been described as an activator of SIRT1 activity (Bonkowski and Sinclair 2016). Some of these effects seem to be due to technical artifacts and should be considered with care (Pezzuto 2019). Nevertheless, SIRT1 activation by resveratrol in vivo was associated with longevity, beneficial effects on metabolic disorders, cardio- and neuroprotection (Fernandes et al. 2017).

Resveratrol has been shown to affect DNA methylation in vitro and in vivo and to reactivate the TSGs *PTEN, BRCA1* and *RASSF1A*, whereas methylation of cell cycle regulators *AURKA* and *CCNB1* was increased at high concentrations. Resveratrol also increased acetylation and activated p53 in prostate cancer cells. Expression of several oncogenic miRNAs was reduced by resveratrol, whereas expression of genes related to apoptosis, cell cycle regulation, cell proliferation and differentiation were predicted to be modulated by upregulation of miRNAs after resveratrol treatment. Pterostilbene similarly was shown to modulate miRNA expression, and it reduced migratory and invasive potential of TNBC cells [review in Lee et al. (2018)].

Kala et al. investigated the influence of a combination of resveratrol (15 μM) and pterostilbene (5 μM) on TNBC cells in vitro (Kala et al. 2015). The combinatorial treatment synergistically reduced cell viability and induced apoptosis in both HCC1806 and MDA-MB-157 TNBC cell lines, but not in MCF10A normal mammary cells. The combined treatment induced G_2/M- and S-phase cell cycle arrest in HCC1806 and MDA-MB-157 cells, respectively, and was more effective than either treatment alone. The combination reduced mRNA and protein expression of the SIRT1 histone deacetylase in both cell lines, leading to reduced SIRT1 activity. This resulted in reduced γ-H2AX as a marker of DNA damage response in combination-treated cells. The combination treatment lowered mRNA expression of DNMTs 1, 3A and 3B as well as DNMT activity. It also lowered mRNA expression of epigenetically regulated hTERT and reduced telomerase activity catalyzed by hTERT (Kala et al. 2015).

In a follow up study, resveratrol and pterostilbene in combination were found to reactivate ERα expression by increasing activating histone marks at the ERα promoter and by reducing DNMT activity and global DNA methylation levels. Combination treatment also re-sensitized the cells to either estrogen (E2, ER-agonist) or 4-hydroxy-tamoxifen (4-OHT, ER-antagonist) treatment, with increased/reduced cell viability after E2/4-OHT treatment and increased/reduced expression of the ER target gene PGR, respectively (Kala and Tollefsbol 2016).

This is another interesting example of combined dietary agents that re-activate ER expression and response to anti-hormonal therapy as a treatment approach for TNBC. Since polyphenols are less bioavailable than more lipophilic compounds

such as SFN and WA and concentrations tested in this study were relatively high, it still needs to be demonstrated that effective doses can indeed be reached in mammary tumors in vivo.

18.2.1.6 Combination of Short Chain Fatty Acid Butyrate with the Demethylating Agent 5-Azacytidine

In the MMTV-neu-Tg mouse model, Her2 (neu) is under the control of the mouse mammary tumor virus (MMTV), and tumors arise from luminal progenitor and basal myoepithelial stem cells (Pathania et al. 2016). Pathania et al. tested a combination of the demethylating agent 5-azacytidine (5-Aza, 1 μg/ml) with the HDAC inhibitor butyrate (1 mM) (further information see Sect. 18.2.2.1). The combination reduced the number of primary and secondary tumorospheres derived from MMTV-neu-Tg tumors more than the compounds alone. The combination also strongly reduced the number and sizes of tumorospheres generated with the human breast cancer cell lines MCF10A4 (basal subtype), CAL51 (triple negative subtype) and the murine cell line 4T1 (triple negative subtype). When 4T1 cells were xenografted into mammary fat pads of Balb/c mice, implantation with tablets releasing 5-Aza (0.5 mg/21 days) and butyrate (10 mg/21 days) significantly prolonged the mean survival time, more than salinomycin (5 mg/kg, intraperitoneal injection), a known cancer stem cell inhibitor (Gupta et al. 2009). Self-renewing cancer stem cells were isolated from MMTV-neu-Tg tumors and cultured in 3D cultures. 5Aza + butyrate led to alterations in mRNA expression of genes enriched in cell cycle and cell division pathways. Downregulated genes were typically upregulated in human breast cancer samples, whereas transcript levels of upregulated genes were generally downregulated in human breast cancer. Among others, three genes with high expression in basal breast cancer (*RAD51AP1, NUSAP1, SPC25*) were downregulated by the combination treatment. *RAD51AP1* is associated with double strand break repair, whereas *SPC25* plays a role during cell division.

Overall, these results demonstrated that 5-Aza in combination with butyrate effectively reduced mammary tumorigenesis and tumorosphere-forming potential of tumor-propagating cells and might be a treatment alternative for basal breast cancer with high levels of cancer stem cells (Pathania et al. 2016).

18.2.1.7 Combination of Oleanane Triterpenoids with the HDAC Inhibitor SAHA

Synthetic oleanane triterpenoids are synthetic analogs of the natural triterpenoid oleanolic acid, which is widely distributed in the plant kingdom with highest concentrations found in olives (*Olea europea* L.) (Ziberna et al. 2017). Oleanane triterpenoids have anti-inflammatory and cyto-protective properties by targeting NRF2/KEAP1, NF-κB, TGF-β, and STAT signaling. These activities have been attributed to their high reactivity with protein thiol groups. Oleanane triterpenoids have been shown to induce cell differentiation and apoptosis and to inhibit cell

proliferation. In vivo, they prevented or inhibited tumor growth in various animal models, especially the development of lung cancer (Liby et al. 2007; Liby and Sporn 2012). CDDO-Me (methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate, also known as bardoxolone methyl or RTA 402) has been tested or is currently undergoing testing in clinical trials for lymphoma (Hong et al. 2012) and pulmonary arterial hypertension (National Library of Medicine 2019). Clinical trials for chronic kidney disease were terminated because of a higher rate of cardiovascular events with bardoxolone methyl treatment compared to placebo treatment (de Zeeuw et al. 2013).

Anti-proliferative and apoptosis-inducing effects of CDDO-Me have been associated with the inhibition of human telomerase reverse transcriptase (hTERT) expression and activity, partly by downregulation of DNMTs and hypomethylation and reduced histone acetylation at the hTERT promoter (Deeb et al. 2014). In a rat model for aflatoxin B1 (AFB1)-induced liver carcinogenesis, CDDO-Im (1-[2-cyano-3-,12-dioxo-oleana-1,9(11)-dien-28-oyl]imidazole) abrogated AFB1-induced miRNA expression (Livingstone et al. 2017).

Tran et al. tested the combination of the HDAC inhibitor vorinostat (also known as SAHA, suberoylanilide hydroxamic acid) with either of the synthetic oleanane triterpenoids CDDO-Me or CDDO-Ea in the MMTV polyoma middle T (PyMT) mouse model of ER-negative mammary tumors (Tran et al. 2013). The combination was more effective than either compound alone in inhibiting pro-inflammatory nitrite production in Raw264.7 mouse macrophages or macrophages derived from the PyMT model, which were stimulated with interferon γ (IFN-γ) or lipopolysaccharides. The CDDO derivatives are potent inhibitors of NF-κB signaling. However, this anti-inflammatory mechanism was not enhanced by co-treatment with SAHA. The combination of SAHA (250 mg/kg diet) with either CDDO-Me (50 mg/kg diet) or CDDO-Ea (400 mg/kg diet) significantly prolonged tumor latency in the PyMT mouse model. This was accompanied by significantly reduced infiltration with tumor-associated marcrophages (TAM). Mechanistically, the combination significantly lowered the secretion of pro-angiogenic MMP9 expression from primary PyMT macrophages and was more effective than the individual compounds. Tissue and plasma levels of SAHA were measured in the range of 50 nM. Therefore, the observed anti-tumorigenic effects might be independent of its known HDAC inhibitory potential (Tran et al. 2013). Reactivation of the ER was not investigated in this study.

In summary, multiple studies have suggested that epigenetic mechanisms are involved in the reduced expression of the ER in TNBC. Several combinations of chemopreventive agents (GTP/EGCG + SFN, resveratrol + pterostilbene, genistein + TSA) led to reactivation of ER in ER-negative tumors and consequent reactivation of anti-estrogen sensitivity. This approach might be a feasible strategy in the clinical management of TNBC, which could be followed up in clinical trials. Also, the combination of chemopreventive agents with clinically approved epigenetic drugs resulted in improved efficacy at low doses in mouse models for breast cancer. Nonetheless, the studies did not prove a causal relationship of the measured effects. Although the combinations were demonstrated to affect epigenetic markers, additional mechanisms might underlie the observed enhancement of tumor latency or reduction in tumor growth and need to be further investigated.

18.2.2 Colorectal Cancer Studies

With 1.1 million estimated new cases and about 550,000 estimated cancer deaths in 2018, colorectal cancer is the third most common cancer type in both the male and female population worldwide (Bray et al. 2018). The great majority of colorectal tumors are adenocarcinomas. Five to 10% of all cases are based on hereditary conditions, including familial adenomatous polyposis (FAP) caused by a mutation in the TSG *APC* (Adenomatous polyposis coli) and non-polyposis colorectal cancer (HNPCC) linked to mutations in DNA repair genes, but the same genetic defects are also involved in the etiology of sporadic cases (World Cancer Research Fund International/American Institute for Cancer Research 2017). Also, chromatin regulators such as *ARID1A* (AT-rich interactive domain 1A), a component of the SWI/SNF chromatin remodeling complex, are frequently mutated in colorectal cancer (Vymetalkova et al. 2019). Driver genes inactivated by DNA hypermethylation include genes involved in Wnt signaling (*APC, SFRP1, SFRP2*), DNA repair (*MLH1, MGMT*), cell-cell adhesion (*CDH1, CDH13, TSP1*), cell signaling (*RASSF1A, RUNX3, ESR1, ID4, IRF8*) and others. Onco-*miR-21* is frequently upregulated in colorectal cancer, whereas the *miR200* family that negatively regulates EMT is frequently silenced by DNA methylation (Lao and Grady 2011).

A large meta-analysis of dietary patterns associated with cancer risk in epidemiological case-control and cohort studies found strong associations between food choice and both decreased and increased colon cancer risk (Grosso et al. 2017). There is increasing evidence that diet affects colonic health and cancer risk through its effects on colonic microbial metabolism, for example through the generation of short chain fatty acids (SCFAs) with HDAC inhibitory activity, but also via alterations in miR expression (O'Keefe 2016; Bultman 2017; Farhana et al. 2018). Most studies investigating combination effects for colorectal cancer prevention accordingly combined butyrate (in vitro) or dietary fiber (in vivo) with another chemopreventive agent (Table 18.2).

18.2.2.1 Combination of Butyrate with Green Tea Catechins

Dietary fiber is fermented by the gut microbiota to short chain fatty acids (SCFAs) including acetate, propionate and butyrate (O'Keefe 2016). In the healthy colon, butyrate is a major energy source for colonocytes (Bultman 2017; den Besten et al. 2013). As a colon cancer preventive agent, butyrate acts by reducing pro-inflammatory and pro-oxidative conditions and was shown to induce cell-cycle arrest, cell differentiation, and apoptosis [review in O'Keefe (2016), Guilloteau et al. (2010), and McNabney and Henagan (2017)]. Many of these activities have been linked to the potential of butyrate to inhibit HDACs, which was discovered almost 40 years ago [summary in Davie (2003)]. Recent studies indicate that butyrate effects on histone-modifying enzymes are concentration-dependent. At low concentrations such as in colonocytes at the base of the colonic crypt, butyrate increased the

Table 18.2 Combination effects in colon cancer models in vitro and in vivo

Model and treatment	Effects	References
In vitro		
HT29, Caco2 EC (100 μM) or EGCG, 20 μM Sodium butyrate 2 mM	Antagonistic activity of GTP on butyrate-induced differentiation ⇩ relocalization of butyrate transporter, independent of HDACi	Sanchez-Tena et al. (2013)
RKO, HT29, HCT116 EGCG 10 μM Butyrate 5 mM	⇧ apoptosis, cell cycle arrest, ⇩ colony formation ⇩ protein expression of HDAC1, DNMT1, survivin, NF-κB p65 ⇧ p21 mRNA and protein expression ⇩ DNA methylation ⇧ H3 hyperacetylation, DNA-damage (γH2AX)	Saldanha et al. (2014)
HCT116 Docosahexaenoic acid (DHA) 50 μM Butyrate 5 mM 5′aza-deoxycytidine 2 μM	⇧ apoptosis ⇩ DNA methylation of apoptosis-related genes: *BCL2l11, CIDEB, DAPK1, LTBR, TNFRSF25*	Cho et al. (2014)
Caco-2, HCT116 SFN or Iberin 6–8 μM Se-methylselenocysteine (SeSMC) or Na-selenite 0.2–5 μM Up to 12 days	⇔ no effect on p16 and *ESR1* promoter CpG islands or LINE1 methylation ⇩ transient reduction of DNMT mRNA levels in Caco2 cells ⇧ transient increase of DNMT mRNA levels in HCT116	Barrera et al. (2013)
In vivo		
Azoxymethane (AOM)-induced colon cancer in Sprague-Dawley rats Fish oil 11.5% + pectin 6% in the diet (FOP) vs. corn oil 15% + cellulose 6% (COC) diet	⇩ tumor incidence by FOP diet ⇧ apoptosis of DNA-damaged colon cells ⇧ anti-apoptotic Bcl2 promoter methylation ⇩ Bcl2 mRNA levels	Cho et al. (2012)
AOM-induced colon cancer in Sprague-Dawley rats Fish oil 11.5% + pectin 6% in the diet (FOP) vs. corn oil 15% + cellulose 6% (COC) diet	⇩ tumor incidence ⇩ inhibition of AOM-induced downregulation of let-7d, miR-15b, miR-107, miR-324-5p and miR-191 expression by fish oil	Davidson et al. (2009)
AOM-induced colon carcinogenesis in Sprague-Dawley rats (colon mucosa, 10 weeks) Fish oil 11.5% + pectin 6% in the diet (FOP) vs. corn oil 15% + cellulose 6% (COC) diet	⇧ miR-19b, miR-26b, miR-27b, and miR-203 expression by FOP diet ⇩ mRNA expression of miRNA targets Ptk2B, Igfr2, Pde4b2, Atp2b1, Tcf4 by FOP diet ⇩ protein expression of Ptk2b, Pde4b2 and Tcf4 by FOP diet	Shah et al. (2011)
AOM-induced colon cancer in Lgr5-EGFP-IRES-creER^{T2} mice Fish oil 11.5% + pectin 6% in the	⇩ number of aberrant crypt foci by FOP diet ⇧ upregulation of putative tumor	Shah et al. (2016a)

(continued)

Table 18.2 (continued)

Model and treatment	Effects	References
diet (FOP) vs. corn oil 15% + cellulose 6% (COC) diet	suppressor miRNAs in colonic mucosa ⇩ downregulation of mRNA expression of predicted target genes upon carcinogen exposure Differential miRNA expression in $Lgr5^{high}$ colonic stem cells vs. $Lgr5^{negative}$ differentiated cells: miR-19b, miR-26b, miR-203	
AOM-induced colon cancer in Sprague-Dawley rats Fish oil 11.5% + pectin 6% in the diet (FOP) vs. corn oil 15% + cellulose 6% (COC) diet	⇩ number of high multiplicity aberrant crypt foci by FOP diet Context-specific alterations in gene expression and chromatin structure ⇧ histone acetylation in AOM-treated FOP diet groups ⇧ combination of fish oil with pectin facilitates DHA-mediated stimulation of nuclear receptors upstream of lipid metabolism genes.	Triff et al. (2018)
AOM-induced colon cancer in Sprague Dawley rats Green tea extract (0.5% in diet) + selenium (1 ppm in diet)	⇩ additive inhibition of large aberrant crypt foci (ACF) and tumor incidence and multiplicity ⇩ DNMT1 expression by green tea extract ⇧ H3 acetylation by selenium ⇩ β-catenin nuclear translocation, cyclin D1 mRNA, proliferation (measured as Ki-67 expression) by selenium	Hu et al. (2013)

⇧ induction, enhancement, increase; ⇩ repression, inhibition, reduction; ⟺ no change

production of acetyl-CoA and stimulated HAT activity, with overall pro-proliferative effects. In colon cancer cells, accumulation of butyrate led to HDAC inhibition, increased acetylation of histones and non-histone proteins such as tubulin and p53, and resulted in inhibition of cell proliferation and cell cycle arrest [summary in Gerhauser (2018) and McNabney and Henagan (2017)]. Additionally, butyrate-mediated inhibition of miRNA expression has been associated with cell cycle arrest and inhibition of metastases [overview in Chen et al. (2019)]. Recently, it has been postulated that SCFA activities might be related to signal transduction through metabolite-sensing G-protein coupled receptors (GPCR) (Tan et al. 2017).

Sanchez-Tena et al. treated HT29 colon cancer cells with butyrate (2 mM) in combination with green tea catechins EC (100 μM) or EGCG (20 μM) (Sanchez-Tena et al. 2013). Interestingly, co-treatment with the polyphenols impaired butyrate-induced differentiation of HT29 cells, measured by alkaline phosphatase activity. This antagonistic effect was independent of HDAC activity. Instead, the green tea compounds inhibited butyrate entry into cells by reducing membrane

localization of the butyrate transporter protein MCT1 (monocarboxylate transporter 1, also known as SLC16A1) (Sanchez-Tena et al. 2013).

Saldanha et al. investigated anti-proliferative potential of the combination of butyrate (5 mM) with EGCG (10 μM) in three colon cancer cell lines (Saldanha et al. 2014). In this study, the combination was more effective than either compound alone in inhibiting cell proliferation. Butyrate + EGCG induced apoptosis and cell cycle arrest and reduced colony formation. Mechanistically, the combination inhibited HDAC activity and induced protein levels of acetylated histone H3, p21, p53 and NF-κB p65. Levels of DNMT1 mRNA and DNMT3A and 3B protein were reduced, and consequently, global DNA methylation was lowered. The combination increased DNA damage, measured as γ-H2AX levels by western blotting and lowered the levels of survivin, which is a negative regulator of apoptosis and often overexpressed in colorectal cancer (Saldanha et al. 2014).

These studies with opposite effects indicate that the applied concentrations and ratio of butyrate and EGCG might influence the outcome of the studies.

18.2.2.2 Combination of Dietary Fiber with Fish Oil

Docosahexaenoic acid (DHA) is a long-chain omega-3 polyunsaturated fatty acid (ω3-FA) from cold-water fish and component of fish-oil. ω3-FAs are essential for human health (Berquin et al. 2008). They are incorporated into cellular membranes and have anti-oxidant and anti-inflammatory activities by activating the NRF2/KEAP1 pathway and reducing the production of pro-inflammatory prostaglandins (Yum et al. 2016; Saini and Keum 2018). In human studies, dietary intake of ω3-FA reduced the risk for chronic degenerative diseases including coronary heart disease, breast cancer and depression (Marventano et al. 2015).

Omega 3-FAs have been shown to target the epigenome at the levels of DNA methylation, histone methylation and miRNA expression [recent review in Lau et al. (2019)]. After intervention of pregnant mothers with DHA in several intervention studies, alterations in DNA methylation were detected in cord blood or blood spots derived from the babies. In vitro cell culture incubation with DHA resulted in downregulation of several HDACs, with potential impact on chromatin structure. DHA also lowered repressive H3K27me3 levels by reducing the protein expression of the histone methyltransferase EZH2 in breast cancer cell lines (Dimri et al. 2010). In addition, DHA treatment led to alterations in miRNA expression, including downregulation of oncogenic *miR-21* in breast and colon cancer, cholangiocarcinoma and neuroblastoma cell lines (Lau et al. 2019).

The group of Robert Chapkin investigated combined effects of pectin (giving rise to butyrate) and fish oil as a source of ω3-FA (fish oil/pectin, FOP diet) in several in vivo colon cancer prevention experiments (Triff et al. 2015). Control animals were fed a diet containing corn oil and cellulose (COC diet), and colorectal carcinogenesis was induced by injection of AOM. Early stages of tumorigenesis were analyzed in colonic mucosa 10 or 16 weeks after carcinogen injection, and tumors were collected after 34 weeks.

In a study by Cho et al. (2012), FOP diet significantly reduced colon cancer incidence and increased the apoptotic index (mean number of apoptotic cells vs. total number of cells per colon crypt) in rats. FOP diet also significantly reduced mRNA expression of the anti-apoptotic regulator *Bcl-2*, concomitant with increased promoter methylation (Cho et al. 2012).

To gain further mechanistic insight, Cho et al. incubated HCT116 cells with butyrate (5 mM) and DHA (50 μM) and measured promoter (de-)methylation of 24 selected apoptosis-related genes (Cho et al. 2014). Global DNA methylation was not altered by either compound or the combination. At a single gene level, butyrate treatment led to demethylation of *BCL2L11*, whereas DHA reduced methylation of the promoter regions of *CITEB, DAPK1* and *TNFRSF25*. All of these pro-apoptotic genes were methylated >60% in untreated control cells. In combination, butyrate and DHA demethylated *BCL2L11, CITEB, DAPK1, LTBR* and *TNFRSF25*. Butyrate alone and in combination with DHA transiently induced mRNA levels of *TNFRS25* and *DAPK1* after 48 h, but significantly reduced transcript levels after 96 h of incubation. Butyrate as an HDAC inhibitor also increased acetylation of histone H3, but there was no additive effect of the combination (Cho et al. 2014).

Davidson et al. studied the impact of FOP diet on the expression of non-coding microRNAs at early stages of tumorigenesis and in tumors (Davidson et al. 2009). Fish oil-containing diets significantly lowered the number of differentially expressed miRNAs in colonic mucosa 10 weeks after AOM injection, and in combination with pectin (FOP diet) significantly reduced tumor incidence. A tumorigenesis-associated reduction of *let-7d, miR-15b, miR-107, miR-324-5p* and *miR-191* expression was prevented by fish oil intervention (Davidson et al. 2009).

In a follow-up study, miRNA expression levels were correlated with mRNA expression of predicted target genes in colonic mucosa 10 weeks after AOM injection. FOP diet prevented the downregulation of *miR-19b, miR-26b, miR-27b*, and *miR-203*, with consequent reduced mRNA expression of the protein tyrosine kinase *Ptk2B*, insulin-like growth factor 2 receptor (*Igfr2*), phosphodiesterase *Pde4b2*, the plasma membrane pump *Atp2b1*, and transcription factor *Tcf4* involved in Wnt signaling. Expression of *Ptk2b, Pde4b2* and *Tcf4* was also reduced at the protein level by the FOP diet (Shah et al. 2011). The antagonistic link between *miR-19b*, *miR-26b* and *miR-203* and their predicted targets *Pde4b2* and *Tcf4* was confirmed in gain and loss of function studies (Shah et al. 2016a).

As further confirmation of these findings, Shah et al. analyzed the influence of fish oil/pectin combination on miRNA/mRNA expression in the stem cell niche of the mouse colonic crypt. As shown previously, FOP diet inhibited AOM-induced ACF by about 37% compared to the COC diet (Shah et al. 2016a). Sorted stem cells with high expression of the stem cell marker *Lgr5* showed elevated expression of *miR-125a-5p*, *miR-190b* and *miR-191* compared to $Lgr5^{low}$ cells. Different from the rat study (Shah et al. 2011), FOP reduced expression of *miR-19b*, *miR-26b* and *miR-203* in Lgr^{high} colonic stem cells of AOM injected animals (Shah et al. 2016b). Expression of the confirmed targets Ptk2b and Tcf4 did not change, and Pde4b was significantly upregulated by FOP diet. These data suggest that the response to AOM and diets might be different in the bulk cell population of the colon vs. stem cells

(Shah et al. 2016a). Also, the data indicated that it is still challenging to causally link miRNA expression changes with expression of target genes.

In a recent study, the spectrum of epigenetic mechanisms was extended to profiling of posttranslational histone modifications (Triff et al. 2018). In confirmation of earlier studies, fish oil-containing diets significantly lowered the number of high multiplicity (HM) aberrant crypts in colonic mucosa 10 weeks after AOM injection. The combination of fish oil with pectin was more efficient than either treatment separately. Gene expression in colonic mucosa was studied by RNA-seq. In AOM-treated animals, FOP diet significantly altered the expression of 83 genes predominantly associated with lipid metabolism (increased fatty acid catabolism, decreased accumulation of lipids, reduction of fatty acid synthesis), whereas fish oil and pectin diets separately altered only 1 or 0 genes, respectively. In the absence of carcinogen treatment, fish oil alone modulated more genes that in combination with pectin (63 vs. 14 genes), whereas pectin alone did not alter expression of any gene. These results indicated that the diets had a context specific effect. The authors also measured activating histone modifications including H3K9ac and H3K4me3 using ChIP-seq in colonic mucosa. In general, they observed poor correlation between the measured changes in histone marks and gene expression. After AOM treatment, none of the diets induced any change in H3K9ac. In the absence of the carcinogen, pectin alone induced more acetylation changes than in combination with fish oil or fish oil alone (24 vs. 15 vs. 4). Conversely, effects on H4K4me3 were stronger in AOM-treated animals than in the absence of the carcinogen and mainly induced by pectin diet (combination: 21 peaks, fish oil: 0 peaks, pectin: 15 peaks, false discovery rate 10%). Key affected pathways were related to metabolic disease, lipid metabolism and cell death and survival. An analysis of upstream regulators identified ligand activated nuclear receptors including PPARs, LXR, FXR, PXR, GCR and HNF4A as main targets. The authors postulated that the combination of fish oil with pectin facilitates stimulation of DHA-ligand activated nuclear receptors associated with lipid metabolism (Triff et al. 2018).

Overall, these studies demonstrated enhanced colon cancer chemopreventive efficacy by the combined intervention with fish oil and pectin and suggest plausible mechanisms of action via modulation of mRNA and miRNA expression and alterations of the chromatin structure, which seemed, however, not directly linked to changes in gene expression.

18.2.2.3 Combination of Isothiocyanates (ITCs) with Selenium

Selenium (Se) is an essential trace element and is incorporated as selenocysteine into selenoproteins, which are involved in redox control and protection from oxidative stress (Barrera et al. 2012; Rayman 2005). Epidemiological studies have associated Se deficiency with increased cancer risk. Supplementation with anorganic sodium selenite in experimental animal studies reduced tumor incidence, but this form of Se might not be representative for dietary organo-Se-compounds. Suggested mechanisms targeted by Se include carcinogen activation, DNA repair, cell proliferation,

apoptosis, angiogenesis, and immune functions (Combs Jr. and Gray 1998; Jung and Seo 2010). Also, Se compounds were shown to affect DNA methylation by inhibition of DNMTs, resulting in upregulation of, e.g., *GSTP1*, *APC* and *CSR1*, in a prostate cancer cell line. Se also reduced HDAC activity and activated gene expression through increase in H3K9 acetylation and miRNA expression, partly through the activity of metabolites (Huang et al. 2011; Barrera et al. 2012). Lack of efficacy or even negative health effects of Se-methionine supplementation in the large Phase III "Selenium and Vitamin E Cancer Prevention Trial" (SELECT) for prostate cancer prevention reduced the hopes for Se as a human cancer chemopreventive agent, although it might still be valuable in geographic areas of Se-deficiency (Lippman et al. 2009; Lu et al. 2016; Vinceti et al. 2014).

Barrera et al. were interested in the combined effects of ITCs and selenium, based on their complementary mechanisms (Barrera et al. 2012). They treated colorectal cancer cell lines in vitro for up to 12 days with SFN or iberin (6–8 μM) in combination with an inorganic (selenite) or an organic (selenium methylselenocysteine, SeMSC) selenium source (0.2–5 μM) (Barrera et al. 2013). In both cell lines, neither single compounds nor the combination affected promoter methylation of *p16, ESR1, APC* and *MGMT*. Also, methylation of the repetitive element *LINE1* was not affected by the interventions. In Caco2 cells, iberin (8 μM) transiently induced mRNA levels of DNMT1, 3A and 3B after 4 days of treatment, whereas SFN treatment (8 μM) rather reduced the expression after 4 and 8 days of incubation. SeSMC had no significant effects and weakened the inhibition by SFN when used in combination. In HCT116 cells, both ITCs transiently induced DNMT1 and 3B mRNA levels after 4 days of culture. DNMT3B mRNA levels were also induced by combinations of both ITCs with either selenium source. Since translation to protein and global methylation changes were not analyzed, it is difficult to draw final conclusions from these observations (Barrera et al. 2013). Overall, the combination of ITCs and Se seemed to result in antagonistic rather than additive effects.

18.2.2.4 Combination of Selenium with Green Tea Catechins

Hu et al. tested selenium (1 ppm as selenium-enriched milk protein in the diet) and green tea extract (0.5% in the diet) individually and in combination to prevent azoxymethane (AOM)-induced colorectal cancer in rats (Hu et al. 2013). Both single interventions reduced the numbers of large aberrant crypt foci (ACF), and there was an additive increase in inhibition in the combination group. Tumor size, incidence and multiplicity was significantly reduced by around 40–50% in the Se diet group. In combination with green tea polyphenols, the Se effect was additively enhanced to 75–80% inhibition of all endpoints. In all groups receiving Se diet, histone H3 acetylation in colonic crypt sections was elevated. Conversely, DNMT1 protein expression was reduced by diets containing green tea extract. Se intervention reduced β-catenin nuclear translocation, Cyclin D1 expression and Ki-67 staining, indicating reduced proliferation (Hu et al. 2013). Overall, the study provided promising results that should be reproduced in additional models and with additional sources of Se.

Table 18.3 Combination effects in other cancer models

Model and treatment	Effects	References
Ovarian cancer		
SKOV3-ip1 (paclitaxel-sensitive), SKOV3TR-ip2 (paclitaxel-resistant) EGCG 20 μM SFN 10 μM	⇧ apoptosis, cell cycle arrest in S and G_2/M-phase ⇧ DNA-damage (pH2AX) ⇩ hTERT protein expression and activity ⇩ Bcl-2 protein levels ⇩ DNMT1 expression	Chen et al. (2013a)
A2780 (cisplatin-sensitive), A2780/CP20 (cisplatin-resistant) EGCG 2.5–40 μM SFN 2.5–20 μM	⇩ cell viability in both cell lines ⇧ cisplatin-induced apoptosis and G_2/M arrest ⇧ p21 expression	Chen et al. (2013b)
Lymphoma		
CA46 EGCG 6–48 μg/ml Trichostatin A (TSA) 3–48 ng/ml	⇩ cell proliferation ⇧ cell cycle arrest in G_0/G_1 and G_2/M ⇩ p16 promoter methylation ⇧ p16 mRNA + protein expression	Wu et al. (2013)
Prostate cancer		
PC3, DU145 Quercetin 5 μM Curcumin 5 μM	⇩ DNMT activity in both cell lines ⇩ DNA methylation at the promoter of the androgen receptor (AR) gene ⇧ reexpression of AR mRNA and protein ⇧ anti-androgen responsiveness ⇧ induction of apoptosis	Sharma et al. (2016)

⇧ induction, enhancement, increase; ⇩ repression, inhibition, reduction; ⟺ no change

18.2.3 Studies with Ovarian Cancer, Prostate Cancer and Lymphoma

Until now, only a few in vitro studies investigated combination effects via epigenetic mechanisms in tumor entities beside breast and colon cancer. Table 18.3 summarized combination studies targeting ovarian and prostate cancer and lymphomas.

18.2.3.1 Combination of EGCG with SFN in Chemotherapy-Resistant Ovarian Cancer Models

Ovarian cancer is the eighth most common cancer in females, with about 295,000 estimated new cases and 185,000 estimated cancer deaths worldwide in 2018 (Bray et al. 2018). Ovarian cancer mortality is relatively high due to a lack of screening options, late diagnosis, and the development of resistance against conventional chemotherapy.

In two studies by Chen et al., the effects of EGCG in combination with SFN were tested in chemotherapy-sensitive vs. resistant ovarian cancer cell lines (Chen et al. 2013a, b). In the first study, the combination of EGCG (20 μM) and SFN (10 μM)

efficiently reduced cell proliferation and induced cell cycle arrest in S and G_2/M phase and apoptosis in paclitaxel-resistant ovarian cancer cells. In addition, expression and activity of hTERT, the catalytic subunit of human telomerase that is often upregulated in cancer cells, as well as expression of the anti-apoptotic *Bcl-2* were reduced (Chen et al. 2013a). Combination of both compounds also effectively reduced DNMT1 expression in the ovarian cancer cell lines. As shown previously, hTERT expression is epigenetically regulated by DNA methylation at specific CpG sites in the promoter region. Loss of methylation at these sites allows interaction with the E2F repressive complex, resulting in hTERT down-regulation [summary in Gerhauser (2013)]. In the study by Chen et al., hTERT promoter methylation was not determined (Chen et al. 2013a).

In the second study, Chen et al. used a pair of cisplatin-sensitive and resistant cell lines to investigate cell growth inhibitory effects of the combination of EGCG (10 μM) and SFN (5 μM) (Chen et al. 2013b). Both compounds in combination reduced cell proliferation and induced apoptosis in both cell lines, and strongly increased sensitivity to cisplatin in the resistant cell line. EGCG + SFN, especially in combination with cisplatin (1.5 μM), also potently induced cell-cycle arrest in G_2/M phase, more than either compound alone. This was linked to up-regulation of cyclin-dependent kinase inhibitor p21 only in the cisplatin-sensitive cell line.

Since these interesting findings were limited to in vitro investigations, further work should demonstrate enhanced efficacy of chemotherapy in combination with EGCG + SFN in animal models for tumor resistance and clinical studies (Chen et al. 2013b).

18.2.3.2 Combination of EGCG with TSA in Lymphoma Cells

Burkitt lymphoma is a rare but highly aggressive subtype of B-cell non-Hodgkin lymphoma (NHL). With an estimated 510,000 new cancer cases and about 250,000 cancer death in 2018, NHL is the eighth most common cancer type in males and the tenth most common cancer type in females worldwide (Bray et al. 2018).

Wu et al. investigated the impact of EGCG (6 μg/ml) and the HDAC inhibitor TSA (15 ng/ml) on proliferation and cell cycle progression of CA46 lymphoma cells via epigenetic regulation of the cell cycle inhibitor $p16^{INK4A}$ (Wu et al. 2013). EGCG in combination with TSA inhibited cell proliferation more potently than either compound alone, and reduced the fraction of cells in S-phase of the cell cycle. The authors demonstrated by methylation-specific PCR that the cell-cycle inhibitor *p16* promoter was demethylated by EGCG in a dose-dependent manner. Co-treatment with TSA enhanced the demethylating effect of EGCG. Reduced methylation at the *p16* promoter was associated with enhanced *p16* mRNA and protein expression, with stronger effects by the co-treatment than with EGCG alone.

This study demonstrated increased cell growth inhibition by combining a demethylating agent (EGCG) with a potent chromatin modulator (TSA). The study focused on investigating the combination treatment effect on cell cycle inhibitor *p16*. It can be assumed that the combination will affect additional targets beside *p16,* which might contribute to the anti-proliferate activity.

18.2.3.3 Combination of Polyphenols Quercetin and Curcumin in Prostate Cancer

After lung cancer, prostate cancer is the second most common cancer types in males, with an estimated 1.27 million new cases and about 360,000 cancer death worldwide in 2018 (Bray et al. 2018). Prostate cancer cell proliferation is driven by androgen binding to the androgen receptor (AR). In a subset of prostate cancer cell lines and prostate tumors, *AR* is silenced by promoter methylation (Massie et al. 2017).

Quercetin-glycosides are flavonoids ubiquitously occurring in fruits, vegetables and beverages (Russo et al. 2012). Quercetin is regarded as a broad-spectrum cancer preventive agent: it has radical-scavenging potential and modulates signaling transduction pathways and transcription factors involved in detoxification, inflammation, cell cycle regulation, apoptosis, angiogenesis, autophagy, immune defense and senescence [reviewed in Kashyap et al. (2019) and Murakami et al. (2008)]. In rodent models, quercetin intervention prevented the development of colon, mammary gland, skin and lung cancers (Murakami et al. 2008). In humans, uptake of quercetin reduced markers of oxidative stress and inflammation (Russo et al. 2012).

With respect to epigenetic mechanisms, activation of HATs and sirtuins by quercetin was linked to anti-inflammatory activity by suppressing COX-2 expression, inhibition of NF-κB acetylation and activation of ERK/JNK signaling. Quercetin led to re-expression of cell cycle inhibitor *p16* through inhibition of promoter hypermethylation and inhibited the histone demethylase LSD1. Quercetin also modulated the expression of several miRNAs in various cancer cell lines [summary in Aggarwal et al. (2015) and Shankar et al. (2016)].

Curcumin (diferuloyl methane) is a yellow pigment found in turmeric (*Curcuma longa*). Curcumin has been used for centuries as a traditional medicine in India and other countries (Gupta et al. 2013). As an anti-inflammatory and cancer preventive agent, curcumin targets NF-κB and other signaling pathways (Pavan et al. 2016; Xu et al. 2018). It induced apoptosis and blocked invasion, metastasis, and angiogenesis in in vitro models, and prevented or inhibited tumor growth in rodent models essentially for all major tumor entities (Gupta et al. 2013; Huminiecki et al. 2017). Curcumin is well tolerated even at high concentrations up to 15 g/day (Gupta et al. 2013; Xu et al. 2018).

With respect to epigenetics, curcumin was shown to inhibit p300/CBP HAT activity and to reduce HDAC and DNMT expression. In addition to histone acetylation, curcumin also inhibited acetylation of non-histone targets such as p53 (Balasubramanyam et al. 2004). Effects on DNA methylation after long-term culture for 240 days with curcumin were interpreted as an indirect effect subsequent to changes in, e.g., NF-κB signaling (Huminiecki et al. 2017). Curcumin and derivatives have also been identified as potent modulators of miRNAs and long noncoding RNAs. Main targets in various tumor entities include *miR-21*/PTEN/Akt signaling, *miR19a/b*/Akt/MDM2/PTEN/p53 signaling, *miR15/16*/Bcl2 or WT1 signaling, and miRNA-mediated effects on EZH2, Wnt signaling, cell viability and apoptosis [extensive reviews in references Huminiecki et al. (2017) and Liu et al. (2019)].

Sharma et al. combined quercetin and curcumin to re-sensitize *AR*-negative prostate cancer cells to anti-androgen treatment by re-expression of AR (Sharma et al. 2016). PC3 and DU145 cells were treated with curcumin (12 μM), quercetin (14 μM) or a 1:1 mix of both compounds (10 μM) at the EC_{25} (effective concentration inhibiting 25% of cell growth). Both compounds alone, but more effectively in combination, reduced DNMT activity in both cell lines, especially after treatment for 72 h. Curcumin alone was more potent than the combination in reducing global methylation levels. The authors demonstrated that a region spanning 27 CpG sites in the AR promoter was methylated in both cell lines, leading to silencing of AR expression at the mRNA and protein level. The combination was significantly more effective in demethylating and re-expressing the AR than either compound alone. Using a luciferase reporter assay, the authors also demonstrated that the combination effectively re-sensitized the cell lines to androgen-stimulation. In both cell lines, the combination was more potent in inducing apoptosis than either compound alone, estimated by FITC-Annexin-V staining and flow cytometry. These findings should be confirmed in animal studies. Since the AR is not commonly deactivated by promoter methylation in human prostate tumors, the results might be limited to the in vitro model and lack relevance for the treatment or prevention of human prostate cancer.

18.3 Summary and Conclusions

The aim of this chapter was to give an overview of mostly diet-derived cancer chemopreventive agents that target epigenetic mechanisms and have been tested in combination to enhance their efficacy. Compounds covered in this chapter include some of the best investigated chemopreventive agents, including green tea catechins, soy isoflavones, quercetin, resveratrol and pterostilbene, the short chain fatty acid butyrate, sulforaphane, selenium, curcumin, synthetic triterpenoids such as CDDO, docosahexaenoic acid and withaferin A.

Many of the combinations have so far only been tested in vitro in cell culture models, with some limitations.

1. Most of the studies based the selection of concentrations used for combination interventions on dose-response analyses using anti-proliferative activity as an endpoint. Concentrations required to target epigenetic mechanisms might differ.
2. One of the main aims of combination studies is to reduce the doses necessary to achieve activity/efficacy. Only few of the studies considered whether the applied concentrations would be achievable in vivo.
3. An aim of combination studies is to combine compounds with complementary activity to enhance the biological effect, preferentially more than additively. To draw conclusions on synergism, additive effects or antagonism, ideally the study design should allow quantitative determination of the mode of interaction. This information is largely missing in the presented studies. A suitable approach is nonlinear modeling, which was reported to be advantageous in comparison to the

frequently used Combination Index according to Chou and Talalay [details in Chou (2010), Boik et al. (2008), and Ashton (2015)].

4. As seen with the combination of GTP and butyrate in colorectal cancer studies, depending on the concentrations tested, the outcome of the combination treatment might differ (Sanchez-Tena et al. 2013; Saldanha et al. 2014). Therefore the likely interactions of the tested compounds should be known to avoid antagonistic effects (DiMarco-Crook and Xiao 2015).
5. Most of the studies focused on preselected target genes. Monitoring of global gene expression changes by RNA-sequencing and gene set or pathway enrichment of differentially expressed genes might help in providing a more complete understanding of the mechanisms of interaction. Additional information might come from genome-wide-omics approaches to monitor changes in chromatin accessibility.

Despite of these limitations, the combination studies have provided interesting results which were often confirmed in animal models. Most of the described projects focused on breast and colorectal cancers. A common theme in breast cancer studies was the reactivation of epigenetically silenced ERα, re-sensitizing ER-negative TNBC to anti-hormonal treatment. Currently, no targeted therapy exists to treat TNBC, and only about 50% of TNBC cases respond to chemotherapy. Therefore, intervention with epigenetically active dietary agents to re-establish sensitivity to anti-hormonal therapy might be a strategy to manage TNBC worth being followed-up in clinical trials. Similarly, for ovarian cancer, increasing the susceptibility of resistant tumors to chemotherapy by co-treatment with epigenetically active compounds such as EGCG + SFN might be a promising strategy which should be further investigated in vivo.

For colorectal cancer prevention, treatment with dietary fiber, which is metabolized to SCFAs affecting chromatin compaction, in combination with additional chemopreventive agents such as DHA, has provided promising results in rodent models (Cho et al. 2012; Davidson et al. 2009; Shah et al. 2011, 2016a; Triff et al. 2018). Advanced –omics technologies including genome-wide DNA methylation analyses, miRNA expression analyses, RNA-sequencing and chromatin immunoprecipitation coupled with sequencing (ChIP-seq) have been employed to allow integrative analyses of multiple epigenetic mechanisms. Overall, these studies have indicated that linking miRNA expression or alterations in DNA methylation and chromatin accessibility with expression of associated target genes is still challenging in dietary intervention studies with weak and infrequent treatment-induced effects, compared to cancer-induced epigenetic alterations. Integration and interpretation of results is aggravated by variation in treatment response observed in individual animals and a lack in statistical power when genome-wide analyses are performed with small numbers of animals per group and results are corrected for multiple testing. Translation of results from animal studies with dietary fiber to the human situation might additionally be complicated by the heterogeneity of the human gut microbiota and varying levels of butyrate-producing bacteria, which adds another level of complexity to human intervention studies with the aim of colon cancer prevention.

In conclusion, combination of epigenetic agents that target the epigenome to enhance efficacy is a promising strategy in cancer chemoprevention. We are beginning to understand how chemopreventive agents affect DNA methylation, histone modifications or miRNA expression in vivo, and how alterations of these epigenetic mechanisms by combination treatments will affect gene expression and can be translated to chemopreventive efficacy. Genome-wide analyses and more systems biology-based approached might be required to fully comprehend the compounds' interactions affecting the epigenome.

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