Cancer Chemoprevention by Targeting the Epigenome

Joseph Huang, Christoph Plass and Clarissa Gerhäuser*

Division Epigenomics and Cancer Risk Factors, German Cancer Research Center, Heidelberg, Germany

Abstract: The term "epigenetics" refers to modifications in gene expression caused by heritable, but potentially reversible, changes in DNA methylation and chromatin structure. Given the fact that epigenetic modifications occur early in carcinogenesis and represent potentially initiating events in cancer development, they have been identified as promising new targets for prevention strategies. The present review will give a comprehensive overview of the current literature on chemopreventive agents and their influence on major epigenetic mechanisms, that is DNA methylation, histone acetylation and methylation, and microRNAs, both *in vitro* and in rodent and human studies, taking into consideration specific mechanisms of action, target sites, concentrations, methods used for analysis, and outcome. Chemopreventive agents with reported mechanisms targeting the epigenome include micronutrients (folate, selenium, retinoic acid, Vit. E), butyrate, polyphenols (from green tea, apples, coffee, and other dietary sources), genistein and soy isoflavones, parthenolide, curcumin, ellagitannin, indol-3-carbinol (I3C) and diindolylmethane (DIM), mahanine, nordihydroguaiaretic acid (NDGA), lycopene, sulfur-containing compounds from *Allium* and cruciferous vegetables (sulforaphane, phenylethyl isothiocyanate (PEITC), phenylhexyl isothiocyanate (PHI), diallyldisulfide (DADS), allyl mercaptan (AM)), antibiotics (mithramycin A, apicidin), pharmacological agents (celecoxib, DFMO, 5-aza-2'-deoxycytidine and zebularine), compounds affecting sirtuin activity (resveratrol, dihydrocoumarin, cambinol), inhibitors of histone acetyl transferases (anacardic acid, garcinol, ursodeoxycholic acid), and relatively unexplored modulators of histone lysine methylation (chaetocin, polyamine analogues, n-3 polyunsaturated fatty acids). Their effects on global DNA methylation, tumor suppressor genes silenced by promoter methylation, histone modifications, and miRNAs deregulated during carcinogenesis have potential impact on multiple mechanisms relevant for chemoprevention, including signal transduction mediated by nuclear receptors and transcription factors such as *NF-κB*, cell cycle progression, cellular differentiation, apoptosis induction, senescence and others. *In vivo* studies that demonstrate the functional relevance of epigenetic mechanisms for chemopreventive efficacy are still limited. Future research will need to identify best strategies for chemopreventive intervention, taking into account the importance of epigenetic mechanisms for gene regulation.

Keywords: Apoptosis, chemoprevention, chemopreventive agents, chromatin modifications, differentiation, DNA methylation, DNA methyltransferase (DNMT), epigenetic, epigenomic, histone acetyl transferase (HAT), histone acetylation, histone deacetylase (HDAC), histone methylation, hypermethylation, hypomethylation, polyphenols, sirtuins.

1. EPIGENETIC PATTERNS IN NORMAL CELLS

Mammals consist of over 200 different tissue-types that are characterized by unique gene expression patterns which undergo drastic changes starting from early embryonic time points all the way to old age. During development, and within each tissue, characteristic gene expression patterns exist that are responsible for the exquisitely specific function of each tissue at a certain developmental stage. While the DNA sequences of an individual in each of these cell types remains identical, this is not the case for epigenetic modifications. Thus epigenetic gene regulation offers additional explanations for regulatory mechanisms involved in temporal and spatial gene expression. Epigenetic modifications include DNA methylation, mainly at cytosines (creating the 5-methylcytosine) when positioned next to a guanine (CpG dinucleotides), histone modifications including methylation, acetylation, phosphorylation, ubiquitination and sumoylation as well as non-coding RNAs that affect the expression of target genes [1]. Epigenetic alterations do not change the genomic DNA sequences and are inherited to daughter cells. Thus epigenetic modifications have the ability to 'fix' altered gene expression patterns that have been established as a consequence of environmental stimuli (e.g. nutrition, chemical exposure, radiation etc.) or as a result of signals in a cells microenvironment and therefore could act as a 'memory' for gene expression patterns.

The interplay of epigenetics marks is now well established, and numerous enzymes and biochemical pathways that participate in the establishment of epigenetic patterns have been identified (reviewed in [2, 3]). For example DNA methylation is mediated by DNA methyltransferases (*DNMT1, 3a*, and *3b*) in the presence of the methyl-group donor S-adenosyl-*L*-methionine. *DNMT3a* and *DNMT3b* have been described as *de novo* methyltransferases that catalyze DNA methylation on DNA sequences not methylated before. *DNMT1* on the other hand is a maintenance DNA methyltransferase that preferentially targets hemimethylated DNA created after replication of a methylated sequence. Thus *DNMT1* assures that DNA methylation patterns can be transmitted to the next generation. Similarly enzymes have been described that establish or remove histone tail modifications (e.g. histone acetyltransferases (HATs) and histone deacetylases (HDACs)) that regulate the acetylation status at unique sites in the histone proteins.

*Address correspondence to this author at the Division of Epigenomics and Cancer Risk Factors (C010), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany; Tel: +49 6221 42 3306; Fax: +49 6221 42 3359; E-mail: c.gerhauser@dkfz.de

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The importance of epigenetic gene regulation has been established for many developmental processes. For example, a female mammalian cell contains two X-chromosomes as compared to one in male cells. The mechanism that compensates for the seemingly double dosage of genes on the X-chromosome is X-chromosome inactivation, first described as highly condensed X-chromosomes visible in the microscope as Barr-bodies [4, 5]. It is now clear that X-chromosome inactivation is mediated by epigenetic mechanisms involving dense methylation of the CpG island sequences on the inactive X-chromosome coupled with histone marks characteristic for silenced gene loci (trimethylation of histone H3 at lysines 9 and 27, H3K9me3 or H3K27me3). Another example is genomic imprinting, a phenomenon that describes the expression of a small number of genes in a parent-of-origin dependent manner [6]. Imprinted genes are either expressed on the paternally-derived allele or the maternally-derived allele. Again it is now well established that gene expression is regulated by imprinting control regions that demonstrate different epigenetic patterns on the two alleles [7]. DNA methylation in these regions is seen on only one allele, associated with repressive marks and a condensed chromosome, whereas the other allele is unmethylated. These imprinting control regions usually regulate clusters of imprinted genes through differential binding of chromatin boundary factors (e.g. *CTCF*) [8].

It is now well established that individuals undergo massive alterations in epigenetic patterns throughout their lifespan [9], starting with a wave of global demethylation in early preimplantation stages all the way to age-related epigenetic patterns [10, 11]. This is best exemplified by the findings of promoter methylation in a significant number of genes; for example in a recent screen of various tissues derived from aging mice, it was shown that 21% of the genes become hypermethylated and 13% show a loss of methylation in the gene-associated CpG island [11]. While the early epigenetic alterations are programmed events, it is likely that age-related alterations are a consequence of environmental factors or cellular milieu which might include stochastic inflammation. Tissue type specific differences have also been described and may have their foundation in different stem cell proliferation patterns or different tissue specific exposures.

2. ENVIRONMENTAL INFLUENCE ON THE EPIGENOME

The reversible nature of epigenetic states allows an organism to respond to changes in the environment. Thus, the epigenome is likely to be changed by environmental modifications and external factors, such as diet and changes in the availability of specific nutrients, exposure to environmental toxins, ultraviolet (UV) radiation and hormonally-active compounds, and even behavioral patterns [12-16]. For example, imposed famine of pregnant women during the Dutch Hunger Winter of 1944-45 led to lower CpG methylation at the imprinted *insulin-like growth factor 2* (*IGF-2*) locus in exposed offspring. The offspring displayed a tendency for glucose intolerance, increased incidence of coronary heart disease, obesity, and breast cancer. In another study, exposure of primates to a maternal high-fat diet increased HDAC activity and modified the chromatin structure in the fetal liver (review in [15]). On the other hand, supplementation with nutrients of the so-called 'one-carbon metabolism' pathway (such as folic acid, vitamin B12, choline and betaine) prevented methylation-associated effects on coat color and obesity in the Agouti mouse model [17]. Embryonic exposure to endocrine disrupting agents, such as the anti-androgenic fungicide vinclozolin, modified DNA methylation in rat testes, and affected sperm function for four generations [17]. Ultraviolet radiation was identified as an environmental carcinogen with "epigenotoxic" effects [16]. DNA methylation was shown to promote UV irradiation-induced DNA damage: methylation at CpG dinucleotides increased the mutation rate induced by UV radiation by promoting the formation of cyclobutane pyrimidine dimers at dipyrimidine sequences with methylated cytosine. This was demonstrated for CpG dinucleotides in the coding region of *p53* during skin carcinogenesis (reviewed in [16]). *In utero* exposure to the synthetic estrogen diethylstilbestrol in a mouse model caused promoter hypomethylation of the estrogen-responsive *lactoferrin* gene in uterine tissue, and increased the incidence for cervical-vaginal carcinoma and uterine fibrosis in humans (review in [15]). Also, *in utero* exposure of mice to a carcinogenic dose of arsenic induced DNA hypomethylation in CG-rich regions and aberrant gene expression in livers of newborn mice. This was associated with a depletion of intracellular methyl group stores through arsenic metabolism. Finally, even patterns of maternal care which are transmitted to the offspring led to modifications in glucocorticoid receptor methylation in the hippocampus of rat pubs (reviewed in [15, 18]).

3. THE CANCER EPIGENOME

3.1. Global DNA Hypomethylation Patterns in Human Malignancies

Fine-tuned DNA methylation patterns exist in every normal tissue and represent the gene expression patterns within each cell type at a given developmental stage. This is not the case in human malignancies. Already over 20 years ago it was reported that the level of 5-methylcytosine is decreased in malignant tissues of several tumor-types [19]. We now understand that loss of DNA methylation (or DNA hypomethylation) is mainly due to loss of methylation in repetitive sequences such as ribosomal DNA repeats, satellite or centromeric repeats that are found heavily methylated in normal tissues but have loose DNA methylation in tumors. Mechanisms for this loss of DNA methylation are unclear but could occur by a passive mechanism which involves DNA replication and inhibition of the maintenance DNA methyltransferase, *DNMT1*. In normal cells *DNMT1* is responsible for the establishment of the DNA methylation patterns on the newly synthesized DNA strands. Alternatively, an active mechanism involving DNA demethylating enzymes is possible (discussed in [20]). Hypomethylation of repeat sequences coincides with chromatin changes that in turn cause genomic instability, a hallmark of cancer genomes, and could even be the initiating event driving tumorigenesis, as shown in a mouse model with reduced *DNMT1* expression [21].

3.2. Cancer-Related Gene Silencing by DNA Hypermethylation

Gain of DNA methylation at normally unmethylated CpG island sequences (known as hypermethylation) is another mechanism by which epigenetic alterations contribute to tumorigenesis. It has been shown now for many cancer-related genes and *bona fide* tumor suppressor genes that epigenetic modifications, preferentially targeting the promoter region of genes, result in gene silencing [22]. Epigenetic silencing of a tumor suppressor gene results in loss of function similar to a genetic deletion or a nonsense mutation. However, epigenetic alterations do not alter the genetic sequence and thus offer the possibility for gene reactivation. Historically, tumor suppressor genes, identified due to the presence of genetic alterations in a cancer have been evaluated for epigenetic alterations in tumors that do not show a gene mutation. The list of such epigenetically silenced genes is extensive and includes genes with functions in for example cell cycle regulation (*retinoblastoma*, $p16^{INK4a}$, $p15^{INK4b}$, $p14^{ARF}$), DNA repair (*MGMT* and *hMLH1*), signal transduction (*RASSF1A*, *APC*), apoptosis (*DAPK1*, *p53*, *caspase-8*), hormone response (*ER, PR, AR, RARβ*), carcinogen metabolism (*GSTP1*), angiogenesis (*maspin, thrombospondin 1*), and invasion or metastasis (*TIMP3, CDH1*) [23, 24].

Colorectal cancer belongs to the best investigated tumor types with respect to alterations in DNA methylation. A series of studies identified genes with increased promoter methylation in normal mucosa with increasing age ('type A' genes), whereas another set of genes was uniquely hypermethylated in cancers or preinvasive lesions ('type C' genes). Importantly, type A genes were described as being more commonly hypermethylated in colorectal cancer (30-100%) than type C genes (10-50%). Type A genes include *ESR1* (*estrogen receptor α*), *CSPG2* (*chondroitin sulfate proteoglycan 2*), *EGFR* (*epidermal growth factor receptor*), *IGF2* (*insulin-like growth factor 2*), *MYOD1* (*myogenic differentiation 1*), *N33* (also known as tumor suppressor candidate 3, *TUSC3*), *PAX6* (*paired box protein 6*), and *RARβ2* (*retinoic acid receptor beta2*). Type C genes include *APC* (*adenomatous polyposis coli*), *CACNA1G* (*Calcium channel, voltage-dependent, T type, alpha 1G subunit*, also known as *Cav3.1*), *CALCA* (*calcitonin-related polypeptide alpha*), *HIC1* (*Hypermethylated in cancer 1*), *MGMT* (*O6-Methylguanin-DNA-Methyltransferase*), *TIMP3* (*tissue inhibitor of matrix metalloproteases 3*), and *WT1* (*Wilms tumor 1*) (summarized in [23]).

In **breast cancer**, the putative tumor suppressor gene *RASSF1A* (*Ras association domain family 1 A*) belongs to the most frequently methylated genes with promoter hypermethylation already detectable in preneoplastic ductal carcinoma *in situ* (DCIS) (review in [23]). Other genes frequently hypermethylated during breast cancer include *ESR1*, *GSTP1* (*glutathione S-transferase π*), *RARβ2* (*retinoid acid receptor β2*), $p16^{INKa}$ (also known as *cyclin-dependent kinase inhibitor 2A*, *CDKN2A*), *DAPK1* (*death-associated protein kinase 1*), *Cyclin D2*, *HIN1* (*high in normal 1*), and *TWIST* (summarized in [24]).

Prostate cancer is characterized by the early and frequent hypermethylation of *GSTP1* in preneoplastic prostatic intraepithelial neoplasia (PIN) and tumor tissue. No genetic alterations (deletions or mutations) have been described for this gene, indicating the important role of epigenetic mechanisms in gene inactivation. Other genes with overall methylation frequency (percentage of hypermethylation detected in all samples analyzed in various studies) greater than 60% include *RASSF1A*, *RARβ2*, *HIC1*, *EDNRB* (*endothelin receptor type B*), *PTGS2* (*prostaglandin-endoperoxide synthase* 2, also known as *cyclooxygenase 2*, *Cox-2*), *MDR1* (*Multidrug-resistance-gene 1*), *ERα* A and B (*estrogen receptor α*), *ERβ*, *Caveolin-1*, and $p16^{INK4a}/p14^{ARF}$ (summarized in [25]).

In **lung cancer**, $p16^{INK4a}$ methylation is detectable at the earliest stages and increases with disease progression. Interestingly, $p16^{INK4}$ hypermethylation is also detectable in normal bronchial epithelium from current and former smokers, and in sputum from high risk individuals and cancer patients. However, *p16* methylation might not be predictive for cancer development, but rather facilitates acquisition of additional genetic and/or epigenetic alterations. Further genes often silenced by hypermethylation early in lung cancer include *DAPK1*, *MGMT* and *FHIT* (*fragile histidine triad protein*) (summary in [23], [26]).

Currently there is great interest to develop these epigenetic modifications as biomarkers for early detection of cancers. They may also represent potential targets for chemoprevention strategies. Sensitive, PCR-based assays have been developed to detect cancer-specific methylation events in body fluids such as sputum, urine or plasma. In recent years, DNA methylation profiling became available, either gel-based [27] or hybridization-based [28-30], and determined that the levels of epigenetically silenced genes is in the order of thousands of genes in a cancer genome [31-34]. Due to the lack of technologies most of the profiling data focuses on CpG island sequences. This however will change with the development of sequencing-based DNA methylation profiling studies as recently shown with selected cell lines [35, 36].

3.3. Histone-Based Epigenetic Modifications During Carcinogenesis: Histone Acetylation and Methylation

Post-translational histone modifications, including histone acetylation and methylation, contribute to epigenetic regulation of gene expression. More than eight different histone modifications are known for taking place mainly at the N-terminal histone tails of histone H3 and histone H4 [37]. These modifications alter the chromatin structure and influence the accessibility of the DNA to transcription factors [38]. Histone modifications are also linked with DNA methylation, as *DNMTs* recruit histone deacetylases (*HDAC*s), leading to histone deacetylation and subsequently to transcriptional repression.

Histone acetyltransferases (*HAT*s) and *HDAC*s are dynamically balanced in regulating the steady state of reversible protein acetylation [39]. **Acetylation of histones** H3 and H4 is associated with active transcription. Addition of acetyl groups leads to an open chromatin structure and active gene expression by allowing transcription factors to access DNA. Consequently, proteins with *HAT* catalytic activity are often transcriptional coactivators, including *GCN5, PCAF, MYST,*

p300/CBP, SRC, and the *TAFII250* proteins [40, 41]. In contrast, removal of acetyl groups from the lysine residues by *HDACs* leads to chromatin condensation and transcriptional repression. Proteins with *HDAC* activity have been classified in four classes [42]. The class I *HDACs*, including *HDACs* 1, 2, 3 and 8, are located in the nucleus, where they interact with transcriptional repressors and cofactors (reviewed in [43]). The class II *HDACs*, including *HDACs* 4, 5, 6, 7, 9 and 10, locate between the cytoplasm and the nucleus. *HDAC* 6 has two catalytic domains, one for histone deacetylation and the other for deacetylation of *tubulin*, and may target non-histone protein substrates [44]. The class III *HDACs* are *sirtuins*, and they dependent on nicotinamine adenine dinucleotide (NAD^+) to remove the acetyl group from lysine residues. Class III *HDACs* target non-histone substrates such as *p53* or *tubulin* (reviewed in [41]). *HDAC* 11 was first classified as a class I enzyme but was reattributed to the class IV *HDAC* [45]. The histone deacetylases of classes I, II and IV have a hydrophobic pocket that allows the acetylated lysine residue to reside, and a zinc ion at the bottom of this catalytic pocket.

Perturbation of the balance between acetylation and deacetylation is a key factor in neoplastic transformation. Aberrant histone acetylation has been linked with several human cancers (reviewed in [39, 43]), for example through inactivating mutations of *p300*, over-expression of *HDACs* in primary human prostate tumors and prostate cell lines, loss of activity of *HATs* or increases in activity of *HDACs*. Cancer cells have high level expression of *HDAC* isoenzymes and a corresponding hypoacetylation of histones, so higher levels of histone acetylation has been described in normal tissue as compared to tumor tissue (lymphoid to lymphomas, and colonic epithelium to colon adenocarcinoma). Reduction in acetylated H4 (ac-H4) has been found in advanced stage tumors, with invasion and lymph node metastasis in gastric and colorectal cancers.

Histone methylation is one of the most-widely described histone modifications which takes place at lysine and arginine residues. The influence on activation and repression of gene expression through lysine methylation is both residue-dependent (K4, K9, K27, K36, K79 in H3) and status-dependent (mono-, di-, and tri-methylation). Methylation of H3K4, H3K36 and H3K79 is associated with transcriptional activation (reviewed in [46]), but the methylation of H3K9, H3K27 and H4K20 are frequently associated with repressed genes. Methylation of histones is also important for checkpoint control following DNA damage [47]. Histone lysine methylation is performed by histone lysine methyltransferases (*HKMTs*) containing a SET domain, that is Suppressor of Variegation (*SUV*) homologs, Enhancer of Zeste proteins (*EZH*), and Trithorax group proteins, and the non-SET-domain DOT1 protein family, using S-adenosyl-*L*-methionine (SAM) as the cofactor [48, 49]. *EZH2* is a component of the multi-protein polycomb-repressive complex 2 (*PCR2*), which functions as a transcriptional repressor through H3K27 methylation. In addition to *HKMTs*, several types of histone demethylases have been identified, *i.e. Lysine Specific Demethylase 1* (*LSD1*) and the *Jumonji domain-containing (JmjC) histone demethylases* (reviewed in [50]).

Aberrant histone modifications may greatly contribute to phenotypic changes during oncogenic transformation, such as lack of differentiation and suppression of growth regulation through cell cycle arrest and apoptosis [50, 51]. Examples include the translocation or altered expression of *HKMTs* including the H3K4, K3K27 and H3K79 methyltransferases, global downregulation of H3K9 and H4K20 methylation, and overexpression of *LSD1*, which drives tumor recurrence through G_2/M transition and cell proliferation during therapy. Moreover, polycomb-repressive complexes *PRC1* and *PRC2* are overexpressed or amplified in cancer, and the methylation levels of H3K27 are enhanced, which are associated with condensed chromatin [52, 53]. Overexpression of *PRC2* and *PRC1* leads to the loss of the *INK4A–ARF* locus in a large number of cancers and prevents the induction of cellular senescence [54].

3.4. Impact of microRNAs on Cancer Development

MicroRNAs (miRNAs) are small non-coding RNAs of 20–22 nucleotides that inhibit gene expression at the posttranscriptional level. They are processed from RNA precursor structures by a complex protein system that includes members of the Argonaute protein family, polymerase II-dependent transcription and the ribonucleases *Drosha* and *Dicer* [55]. MiRNAs regulate the transformation of mRNA into proteins, either by imperfect base-pairing to the mRNA 3'-untranslated regions to repress protein synthesis, or by affecting mRNA stability. To date, the miRBase database lists about 1000 human miRNAs, and each miRNA is expected to control several hundred genes. MiRNAs are involved in the regulation of key biological processes, including development, differentiation, apoptosis and proliferation, and are known to be altered in a variety of chronic degenerative diseases including cancer [56]. They have been implicated in cancer initiation and progression, and their expression is often down-regulated during carcinogenesis. Deregulation of specific miRNA has been associated with certain tumor types, but this information is far from being conclusive, and the list is growing every day. By aligning the nucleotide sequence, targets of miRNA can be predicted by database searches using software tools (such as TargetScan, MirWalk), even though many of these targets have not been validated yet. Major mechanisms of miRNA deregulation include genetic and epigenetic alterations as well as defects in the miRNA processing machinery [57, 58]. In addition, human environ-mental carcinogens such as cigarette smoke have been found to effect the microRNA system [59].

4. CHEMOPREVENTIVE AGENTS TARGETING THE EPIGENOME

4.1. Development of a New Paradigm

Epigenetic changes have been identified as some of the earliest events observed during carcinogenesis and therefore represent interesting novel targets for cancer chemoprevention [23]. As an example, hypermethylation of CpG islands may precede the neoplastic process and continuously increases from early preneoplastic lesions to invasive cancer. This has been demonstrated for various malignancies,

Table 1. Overview of Chemopreventive Agents Targeting Major Epigenetic Mechanisms

Compounds	DNA methylation	Histone modifications	miRNA
Micronutrients and vitamins			
Folate	[70, 81-85]		[86, 87]
Organoselenium compounds:	[92]		
NaSelenite	[93-96]	[96]	[98]
Benzyl selenocyanate (BSC), p-XSC,	[92]		
α-Methylselenopyruvate (MSP), α-Keto-γ-methylselenobutyrate (KSMB)		[99]	
Retinoic acid (RA)	[108-111, 114]	[108, 110-112, 293]	[106, 107]
Vit. E	[118]		[117]
NaButyrate and resistant starch (pre-biotic)	[125, 126]	[39, 294, 295]	[127, 296]
Bifidobacterium lactis (pro-biotic)	[126]		
Polyphenols and other natural products			
EGCG, green tea polyphenols	[129-131, 133-136, 138, 139, 142, 146, 297]	[147]	[148]
Apple polyphenols	[140]		
Coffee polyphenols: caffeic acid, chlorogenic acid	[132]		
Dietary polyphenols: rosmarinic acid, ellagic acid, baicalein	[298]		
Genistein, soy isoflavones	[125, 163, 164-167, 173-177]	[159-162, 166, 299]	[169-172, 197]
Parthenolide	[178]		
Curcumin	[188]	[184-186]	[189-191]
Ellagitannin			[193]
Indol-3-carbinol (I3C), diindolylmethane (DIM)			[170, 197, 198]
(Oltipraz, 5,6-benzflavone, N-acetylcysteine)			[198]
Mahanine, Mahanine derivative	[200, 300]		
Nordihydroguaiaretic acid (NDGA)	[205-207]		
Lycopene	[164]		
Sulfur-containing compounds			
Sulforaphane (SFN)	[225]	[221, 222, 224-228]	
Phenylethyl isothiocyanate (PEITC)	[229, 231]	[230]	[232, 233]
Phenylhexyl isothiocyanate (PHI)	[234]	[234-236, 238, 289]	
Diallyldisulfide (DADS)		[244, 245]	
Allyl mercaptan (AM)		[220, 243]	
Antibiotics			
Mithramycin A	[247]		[248]
Apicidin		[249, 250]	
Pharmacological agents			
Celecoxib, DFMO	[257]		
5-Aza-2'-deoxycytidine	[65, 94, 114, 301-304]		
Zebularine	[66, 304]		
Compounds affecting SIRT activity			
Resveratrol		[264]	[270, 271]
Dihydrocoumarin (DHC)		[272]	
Cambinol		[273]	
HAT inhibitors			
Anacardic acid		[275-277]	
Garcinol		[279, 280]	
Ursodeoxycholic acid (UDCA)		[283]	
Modulators of histone lysine methylation			
Chaetocin		[286, 287, 305]	
Polyamine analogue PG11144		[288]	
n-3 Polyunsaturated fatty acid (n-3 PUFA)		[291]	[292]

including breast, colon, prostate, lung, esophageal, cervical and gastric cancer, and myelodysplastic syndrome [60, 61], and can be recapitulated in animal models for cancer progression [62, 63]. Consistently, epigenetic drugs such as 5-aza-2'-deoxycytosine and zebularine were effective in inhibiting tumor formation in genetically- or carcinogen-induced rodent models for cancers in the intestine, lung, prostate, and oral cavity, and a murine melanoma xenograft model (summarized in Supplementary Table **1**). Both 5-aza-2'-deoxycytosine and zebularine were tested in the $APC^{Min/+}$ model that has been extensively utilized for colon cancer prevention studies [64]. 5-Aza-2'-deoxycytosine inhibited adenoma numbers by more that 95% when intervention was started at the age of 7 days, whereas it was ineffective when the intervention was delayed by 50 days [65]. These data point to early epigenetic events targeted by the intervention. Zebularine also strongly reduced adenoma numbers in $APC^{Min/+}$ female mice >95% with intervention starting at 7 days of age [66], indicating that the timing of intervention might be an important determinant of efficacy.

In contrast to tumour suppressor genes irreversibly inactivated by genetic alterations, genes silenced by epigenetic modifications are still intact and can be reactivated by small molecules or dietary factors acting as modifiers of epigenetic mechanisms. To foster research on interactions of diet and DNA methylation and their influence on cancer prevention a coordinated initiative was launched by the Division of Cancer Prevention at the National Cancer Institute (Bethesda, USA) [23, 61, 67, 68]. Over the last few years, evidence has accumulated that natural products and dietary constituents with chemopreventive potential have an impact on DNA methylation and chromatin remodeling [41, 69-75]. Only recently do we begin to understand their influence on miRNA expression and functional consequences, but it can be anticipated that miRNA-related mechanisms will become an important area in chemoprevention research in the future [76-79].

The aim of this review is to provide a comprehensive overview of chemopreventive agents targeting all aspects of epigenetic gene regulation, including DNA methylation, histone modifications and miRNAs. An overview of all agents and epigenetic mechanisms that they affected is provided in Table **1**. Chemical structures of compounds that influence DNA methylation and miRNA expression are depicted in Fig. (**1**), whereas structures of compounds influencing the chromatin as a result of modifications in histone acetylation and methylation are provide in Fig. (**2**). We have include information of micronutrients and vitamins, butyrate, polyphenols from various sources, selenium and sulfur-containing compounds and other bioactive dietary components, antibiotics, and pharmacological agents. The following sections will provide a short description of the chemopreventive potential of these compounds demonstrated in *in vitro* investigations, rodent carcinogenesis models, and epidemiological studies, followed by a summary of their epigenetic effects. Detailed descriptions on utilized methodologies and results of studies on DNA methylation, histone acetylation, histone methylation and miRNAs *in vitro*, *in vivo* and in human studies have been collected in Supplementary Tables **2-11**.

4.2. Micronutrients and Vitamins

4.2.1. Folate

Folate, a water-soluble vitamin of the B-family, plays an important role in one-carbon metabolism, which provides methyl groups for methylation reactions. Folate is an essential factor for maintenance of DNA biosynthesis and DNA repair. Folate-deficiency leads to global DNA hypomethylation, which was linked to genomic instability and chromosomal damage. Recent data indicate that a diet deficient in folate and other methyl-group donors modulates the profile of miRNA expression [76]. Epidemiological studies revealed an inverse relation of folate status and the risk for colorectum, breast, ovary, pancreas, brain, lung and cervix cancer [80-82]. As an essential micronutrient, it has to be provided with the diet. Important sources include citrus fruits, dark-green vegetables, whole grains, and dried beans. Alcohol misuse is often associated with folate deficiency.

Over the last decade, the relationship between folate status, DNA methylation and cancer risk has been analyzed in numerous rodent carcinogenesis models and in human intervention studies. Overall, the results are inconclusive and depend on various parameters, for example the severity of folate deficiency, dose- and timing of the intervention, and on health status (review in [70, 81-84]). Recent data even indicate that an excessive intake of synthetic folic acid (from high-dose supplements or fortified foods) may increase human cancer risk by accelerating growth of precancerous lesions [82]. Therefore, folate-deficiency should be prevented by dietary intake, but supplementation can not be generally recommended.

To identify dietary factors associated with protection from DNA promoter hypermethylation in exfoliated aero-digestive tract cells, Stidley *et al.* performed a cohort-based observation study with 1100 participants. Promoter methylation of eight candidate genes frequently methylated in lung cancer and associated with cancer risk was analyzed in sputum samples from current and former smokers and related to the intake of various dietary factors. Significant protection from DNA hypermethylation was observed for regular consumption of folate (OR (odds ratio) = 0.84 per 750 µg/d; CI (95% confidence interval), 0.72-0.99), leafy green vegetables (OR, 0.83 per 12 monthly servings; CI, 0.74-0.93), and multivitamin use (OR, 0.57; CI, 0.40-0.83) [85].

It is maybe not surprising that folate status also influences miRNA expression. Rats fed a folate- and methyl-deficient diet develop hepatic preneoplastic lesions after 36 weeks and hepatocellular carcinoma after 54 weeks. miRNA profiles were analyzed in tumor tissue. Compared to liver samples of age-matched control rats, tumors showed increased expression of several miRNAs including *let-7a* and *miR-21*, and reduced expression of liver specific *miR-122*. Replenishment of folate starting at 36 weeks normalized the expression of *miR-122*, and prevented tumorigenesis [86]. In cultured human lymphoblastoid cells, folate deficiency resulted in a global increase in miRNA expression. Interestingly, miRNA up-regulation was completely reversible by changing to control medium with adequate folate levels. *miR-222* was identified as significantly overexpressed under

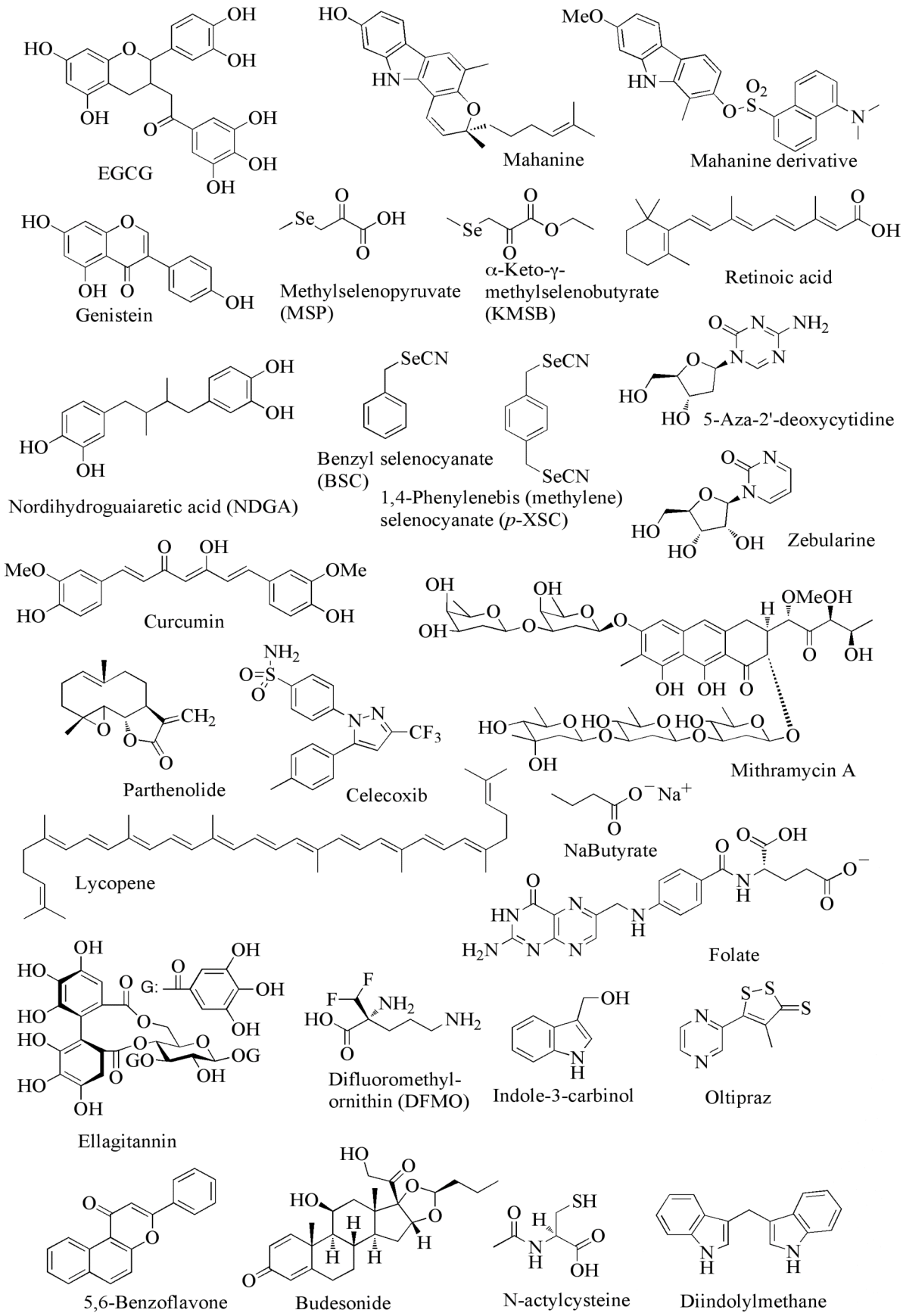

Fig. (1). Chemical structures of chemopreventive agents targeting DNA methylation and miRNAs.

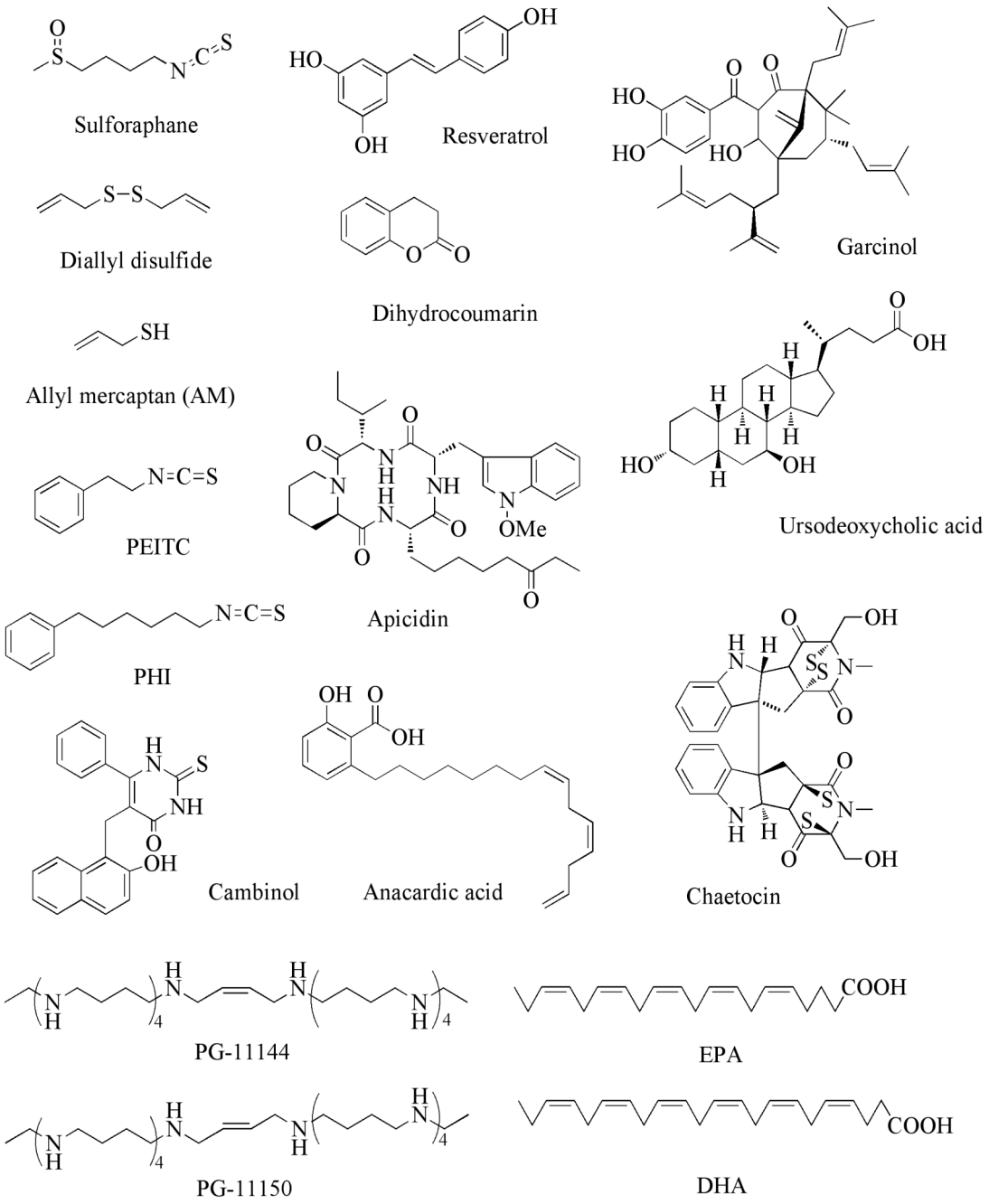

Fig. (2). Chemical structures of chemopreventive agents targeting histone modifications.

folate-deficient conditions, and this was also observed in human peripheral blood cells of individuals with low folate intake [87]. These data indicate that miRNA expression is directly linked to the nutritional status in human individuals.

4.2.2. Selenium Compounds

Selenium (Se) represents an essential trace element and micronutrient and is incorporated as selenocysteine into selenoproteins. Selenoproteins such as glutathione peroxidases and thioredoxin reductase confer protection from oxidative stress and control cell redox status [88]. Dietary selenium occurs as Se-methionine (SM), Se-cysteine or Se-methyl-Se-cysteine (SMC). Insufficient Se supply in many parts of the world has been associated with increased cancer risk, and Se supplementation, especially under Se-deficient conditions, is associated with lower tumor incidence. Most experimental animal studies for cancer prevention have been performed with the anorganic salt sodium selenite (NaSelenite) as source of Se, and may not be directly extendable to dietary organo-Se-compounds. Doses for cancer preventive effects were usually higher than those required for optimal selenoprotein activity, therefore additional suggested mechanisms for cancer prevention by Se may include its effects on cell growth, apoptosis, DNA repair, control of tumor angiogenesis, inhibition of carcinogen activation and

immune function [89]. Enthusiasm for Se as a human cancer chemopreventive agent has been dampened by the lack of efficacy of Se-methionine supplementation in the large Phase III "Selenium and Vit. E Cancer Prevention Trial" (SELECT) on prostate cancer prevention [90]. Null result may be due to the fact that participants in this study were not Se-deficient, and therefore did not gain benefit from the intervention [91].

NaSelenite and organo-Se-compounds target the epigenome by multiple mechanisms. An early study by Fiala *et al*. demonstrated that NaSelenite and two synthetic chemopreventive seleno-compounds, benzylselenocyanate (BSC) and 1,4-phenylenebis(methylene)selenocyanate (p-XSC) [89] inhibited *DNMT* activity *in vitro* with IC_{50} values of 3.8, 8.1 and 5.2 μM, respectively [92]. The following series of studies by Davis and Uthus focused more on prevention of global DNA hypomethylation. NaSelenite was shown to increase global DNA methylation in Caco-2 and HT29 human colon carcinoma cells and in colon and livers of rats, in comparison with animals on a Se-deficient diet [93]. In carcinogen-treated rats, NaSelenite supplementation reduced colonic aberrant crypt–formation (ACF) and lowered the SAM/SAH ratio compared to Se-deficient animals. The relation between global DNA methylation and colon carcinogenesis in this model however appears to be quite complex. Intervention with 5-aza-2'-deoxycytidine (as a positive control substance) reduced global DNA methylation and the incidence of ACF, whereas Se-deficiency reduced global DNA methylation, but increased the numbers of ACF [93-95]. Therefore, the authors concluded that changes in DNA methylation may not be the mechanism responsible for the chemopreventive effect of dietary selenium [94]. A more recent study by Xiang *et al.* has demonstrated that reactivation of epigenetically silenced genes by NaSelenite in LNCaP cells involves a dual effect on both DNA methylation and histone modifications. NaSelenite treatment lowered *DNMT* mRNA and protein expression, reduced global DNA methylation, and led to the re-expression of *GSTP1*, *APC* and *CSR1*. This was associated with reduced *GSTP1* promoter methylation. Also, it inhibited *HDAC* activity and increased ac-H3 levels, but lowered methylation at H3K9. Consequently, ac-H3 binding to the *GSTP1* promoter was increased, whereas the association with *DNMT1* and repressive H3K9me2 was reduced [96]. In a study further elucidating the apoptosis-inducing mechanisms of NaSelenite in LNCaP cells, NaSelenite treatment triggered a rapid activation of *p53* as well as *p53*-target genes including *miRNA 34* [97]. *miR-34b* and *miR-34c*, but not *miR-34a* expression rapidly increased within 4-8 h, suggesting that the NaSelenite-induced growth arrest and anti-cancer activity is mediated in part *via* a miRNA component. Interestingly, *miR-34* has been identified as a putative tumor suppressor gene, which is often epigenetically silenced in tumor cells. *miR-34b/c* targets include many proteins involved in the regulation of apoptosis, G_1-arrest, senescence and inhibition of migration (reviewed in [98]).

Dietary selenium sources such as Se-methyl-Se-cysteine (SMC) and Se-methionine (SM) can be metabolized to α-methylselenopyruvate (MSP) and α-keto-γ-methylselenobutyrate (KMSB), which structurally resembles the *HDAC* inhibitor sodium butyrate [99]. This prompted Nian *et al*. to investigate *HDAC*-inhibitory potential of these α-keto acid metabolites. *In vitro* assays with human *HDAC1* and *HDAC8* showed that although the parent compounds SM and MSC had little effects on *HDAC* activity at a concentration of 2 mM, MSP and KMSB caused a dose-dependent inhibition of *HDAC1* and 8. Enzymatic kinetics and computational modeling supported a mechanism that identified MSP as a competitive inhibitor of *HDAC8*, involving reversible interaction with the active site zinc atom. In human colon cancer cells, ac-H3 levels were increased, accompanied with dose-dependent inhibition of *HDAC* activity. Also, MSP and KMSB induced *p21* levels, cell cycle arrest at G_2/M phase, and apoptosis [99]. Further research is required to investigate interactions between these epigenetic mechanisms induced by selenium compounds.

4.2.3. Retinoic Acid (RA)

The most biologically active metabolite of Vitamin A (retinol) is retinoic acid (RA). Vitamin A is an essential dietary component and is required for normal development, growth and differentiation of epithelial cells, immune functions, reproduction, bone formation, hematopoiesis, and night vision [100, 101]. Epidemiological evidence indicates an inverse relationship between Vitamin A intake and cancer risk. Therefore, chemopreventive activity of RA and structurally related retinoids has been intensively investigated. They suppress tumor development in carcinogenesis models for skin, breast, oral cavity, lung, prostate, bladder, liver and pancreatic cancer [102]. In several clinical studies, retinoids were applied to individuals at increased risk of developing cancer, for example those with premalignant lesions or at risk to develop a second primary tumor (summarized in [102, 103]).

The effects of RA and other retinoids are mediated by nuclear retinoid receptors that belong to the steroid hormone receptor superfamily, namely *RARα, RARβ* and *RARγ*, and *RXRα, RXRβ* and *RXRγ* (further description in [101]). Alterations in retinoic acid receptors may contribute to cancer development. For example, acute promyelocytic leukemia (APL) is caused by a translocation of *RARα* resulting in a fusion protein, usually with the *promyelocytic leukemia* (*PML*) gene. This fusion protein *PML-RARα* acts as a co-repressor at target promoters and results in repression of RA-responsive gens. This defect is efficiently treated by differentiation therapy with RA. Some RA-resistant leukemia cells failed to respond to RA alone [104], but the treatment of RA-refractory APL blasts with RA plus *HDAC* inhibitors, as well as demethylating agents, restored RA sensitivity and cell differentiation [105]. Data by Garzon *et al.* now suggest that miRNAs are involved in the RA-induced granulocytic differentiation of APL. Using a microarray approach, they identified a series of nine miRNAs upregulated by RA treatment of NB4 acute promyelocytic leukemia cells and in RA-treated primary blasts of leukemia patients. These miRNAs included *miR-15*, *miR-16* and several members of the *let-7* family with predicted roles as tumor suppressors and confirmed targets involved in hematopoietic differentiation and apoptosis [106]. Rossi *et al.* further extended these studies to long non-protein coding (nc) RNAs. They analyzed the expression of 243 miRNAs and 492 long non-protein coding RNAs during RA-induced differentiation in NB4 cells using a Ribochip approach. Several deregulated miRNA from earlier studies

were confirmed to be differentially expressed by RA-treatment. In addition, 58 long ncRNAs showed changes in expression after RA treatment, but their function in proliferation and RA-induced differentiation has not been further investigated yet [107].

RARβ expression is often reduced or silenced in tumor cells, and epigenetic mechanisms including promoter methylation play a predominant role in its inactivation. Therefore, combination of RA with natural or synthetic *DNMT* or *HDAC* inhibitors to facilitate reexpression of *RARβ* may provide beneficial effects for chemoprevention in RA-unresponsive premalignant lesions [100]. Loss of expression of the *RARβ2* tumor suppressor gene is commonly observed during breast carcinogenesis. RA therapy failed to induce *RARβ2* in primary breast tumors if a methylated *RARβP2* promoter was present [108]. DNA methylation leads to repressive chromatin at *RARβP2*. By inducing an appropriate level of histone re-acetylation at the *RARβP2* promoter, endogenous *RARβ2* transcription was reactivated from unmethylated, as well as from methylated *RARβP2* in breast cancer cell lines and xenograft tumors. Di Croce *et al*. detected a reduction of *RARβ2* promoter methylation linked with *RARβ2* mRNA re-expression in NB4 cells after RA treatment [109]. However, in the same cell line, Nouzawa *et al.* were unable to find RA-mediated alterations in DNA methylation using a CpG islands microarray approach. When specifically analyzed, *RARβ* CpG island methylation was not influenced by RA treatment in this study. However, over 100 CpG islands within 1 kb of transcription start of a known human gene became hyperacetylated following RA-induced differentiation. One CpG island associated with the *HoxA1* gene was aberrantly methylated in NB4 cells, but became hyperacetylated after RA treatment. This indicated that *HoxA1* might be a new target of RA in APL [110].

RA treatment induces a differentiated phenotype of human teratocarcinoma HT cells, human acute myeloid leukemia HL60 cells [111] and human breast cancer SK-BR-3 cells [112]. In HL-60 and HT cells, RA-induced cell differentiation was accompanied by progressive histone hypoacetylation coupled with a gradual accumulation in *hTERT* promoter methylation, reduced *hTERT* expression and lower telomerase activity [111]. The *hTERT* promoter was less methylated in pluripotent HT cells than in multipotent HL60 cells throughout a 12-day differentiation process. This origin-dependent epigenetic change was also confirmed in histone acetylation studies, indicating that the *hTERT* promoter was more resistant to deacetylation in HT cells than in HL60 cells. However, *hTERT* methylation was not influenced by RA treatment in SKBR3 breast cancer cells [113].

Tang *et al.* provide first indication that epigenetic mechanisms might contribute to RA-mediated chemopreventive effects *in vivo*. They investigated the effect of RA at two concentrations alone and in combination with the *DNMT* inhibitor 5-aza-2'-deoxycytidine on carcinogen-induced oral cavity carcinogenesis in mice. Both compounds alone and in combination reduced the average number of oral lesions per mouse; combined treatment additionally reduced tongue lesion severity. *RARβ2* mRNA expression, reduced in tongue tissue by carcinogen treatment, was non-significantly increased by intervention with both compounds, whereas *Cox-2* and *c-Myc* mRNA expression, induced by the carcinogen, was lowered by the intervention [114].

4.2.4. Vitamin E (Vit. E)

Vitamin E (α-tocopherol and structurally related tocopherols and tocotrienols) is found at high concentrations in vegetable oils. It acts as a direct scavenger of reactive oxygen species, and contributes indirectly to intracellular anti-oxidant defense by inducing the activities of anti-oxidant enzymes and glutathione (GSH) synthesis. Epidemiologic observations suggest an inverse association between dietary Vit. E intake and risk of cancer, but the results of intervention studies in humans are not conclusive (comprehensive review in [115]). Preclinical testing demonstrates cancer preventive activity in rodent models for breast and prostate cancer. Beside anti-oxidant activity, Vit. E acts by anti-inflammatory mechanisms, modulates nuclear receptor signaling and inhibits cell growth by induction of apoptosis. Tocotrienols possess stronger anti-cancer activity than α-tocopherol, and their efficacy is due to mechanisms beside anti-oxidant activity [115, 116].

The influence of Vit. E on epigenetic mechanisms was analyzed in rats maintained for 6 month on a Vit. E-deficient or control diet. Vit. E-deficiency increased hepatic mRNA expression of *5-α-steroid reductase type 1* (*SRD5A1*) and reduced mRNA levels of the regulatory subunit of *γ-glutamyl-cysteinyl synthase* (*GCLM*). Also, Vit. E deficiency significantly reduced the expression of *miR-122* and *miR-125b* which play a role in lipid metabolism, inflammation and cancer progression, respectively [117]. Fischer *et al.* argued that Vit. E deficiency may influence DNA methylation by limiting the availability of S-adenosyl-*L*-methionine *via* its influence on GSH synthesis. They investigated global and specific DNA methylation levels in hepatic DNA. However, Vit. E status did not affect global DNA methylation levels. In addition, changes in the expression of *SRD5A1* and *GCLM* mRNA were not associated with changes in promoter methylation of both genes [118].

4.3. NaButyrate

Butyric acid (or it sodium salt NaButyrate) is a major short-chain fatty acid produced by colonic fermentation of resistant starch and dietary fiber. It serves as a major energy source for intestinal epithelial cells. The potential of butyrate to prevent colon carcinogenesis has been intensely investigated and is associated with inhibition of inflammation and oxidative stress, induction of cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes, and decreasing the transformation of primary to secondary bile acids as a result of colonic acidification [119, 120]. Butyrate has been reported to increase proliferation in normal colonocytes, in contrast to its effects on colon cancer cells. This 'butyrate paradox' has recently been reviewed [121]. Epidemiological studies indicate an inverse relationship between dietary fiber intake and colon cancer risk, but the results are still inconclusive. The lack of consistent correlation may partly been influenced by the reduced expression of the butyrate transporter *SMCT1* (*sodium-coupled monocarboxylate transporter 1*) in human colon cancer, which is due to epigenetic silencing by promoter methylation.

More than 30 years ago, butyrate was first described to inhibit *HDAC* activity *in vitro* and in cell culture and to cause rapid histone hyperacetylation in Friend leukemia cells [122-124]. Many of its effects on gene expression and its anti-proliferative activity are likely related to changes in chromatin structure. Other potential cellular targets include acetylation of non-histone proteins, alteration of DNA methylation, inhibition of histone phosphorylation, and modulation of intracellular kinase signaling [120]. One recent report indicates that butyrate induces *RARβ2* promoter demethylation and reactivation in colon cancer cells, and enhanced responsiveness to RA. Demethylation by butyrate apparently was not genome-wide and independent of DNA replication [125]. In a small human intervention trial with resistant starch alone or in combination with the probiotic *Bifidobacterium lactis,* methylation changes at 16 CpG island loci were analyzed in rectal biopsies of male and female healthy individuals. Only *MINT2* methylation was associated with a significant treatment effect; these results should be interpreted with caution [126].

A recent study investigated the effect of butyrate on miRNA expression. During butyrate-induced differentiation of embryonic stem cells to hepatocytes, 17 miRNAs were upregulated, and 22 and 27 miRNAs were downregulated 6 and 9 days after first treatment (abstract only [127]).

4.4. Polyphenolic Compounds and other Natural Products

4.4.1. (-)-Epigallocatechin gallate-3-gallate (EGCG), Green Tea Polyphenols (GTP), and Polyphenols from other Dietary Sources

Green tea polyphenols (GTP) are a mixture of flavan-3-ols characterized by a catechol moiety. The main GTP is (-)-epigallocatechin gallate-3-gallate (EGCG); further catechins include epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC). EGCG and GTP have demonstrated cancer preventive activity in animal carcinogenesis models of all major organ sites, including lung, colon, breast, prostate, skin, stomach, and liver cancer and tumors of the oral cavity (summarized in [128]). Results from human epidemiological studies are less conclusive; this was attributed to low quantities of tea consumed. *In vitro*, EGCG and GTP target multiple mechanisms for chemopreventive activity, albeit mostly at higher concentrations than usually detectable in human or in rodent models. EGCG acts as a pro- and antioxidant, triggers signal-transduction pathways, and inhibits enzyme activities, receptor-dependent signaling cascades, and angiogenesis.

In 2003, Fang *et al.* were first to describe an influence of EGCG on DNA methylation [129]. This seminal report initiated research on the impact of other polyphenols and chemopreventive agents on DNA methylation and additional epigenetic mechanisms. Potential of EGCG, GTP and other catechins and flavonoids to inhibit the enzymatic activity of human or bacterial *DNMT in vitro* has been investigated in several studies ([130, 131], summarized in [74]). Fang *et al*. postulated direct binding of EGCG to the catalytic pocket of *DNMT* based on *in silico* modeling. Using nuclear extracts as an enzyme source, poly(dI-dC)·poly(dI-dC) as a substrate, and radioactive detection, they suggested that EGCG is a competitive inhibitor of *DNMT* activity with respect to the substrate poly(dI-dC)·poly(dI-dC) [129]. Studies by Lee *et al*. indicate that the mechanism of *DNMT* inhibition by catechins, flavonoids and coffee polyphenols might also be indirect [130, 132]: Tea polyphenols and other flavonoids with a catechol moiety are methylated by the endogenous enzyme *catechol-O-methyltransferase* (*COMT*). This methylation step results in the conversion of S-adenosyl-*L*-methionine (SAM) to S-adenosyl-*L*-homocysteine (SAH). As a consequence, *DNMT* activity can be influenced in two ways: SAH is a negative feedback regulator of methyl-transferases including *DNMTs* and *COMT*; also, catechol-methylation by *COMT* may lead to a depletion of SAM. Lee *et al.* demonstrated that Mg^{2+} enhances the potential of EGCG and the flavonoid myricetin to inhibit human *DNMT*, independent of *COMT*. This was attributed to a stabilization of the binding interaction between Glu1265, a key catalytic site residue of human *DNMT1*, and the gallic/pyrogallic acid moiety of EGCG and myricetin, respectively [74, 130]. Depending on the assay conditions (human or bacterial enzyme, nuclear extracts or recombinant enzymes as enzyme source, presence of *COMT*, choice of substrate, incubation times, buffer composition, mode of detection), results from *in vitro* studies with GTP and other polyphenols may not always be comparable [74].

In addition to inhibition of *DNMT* activity *in vitro*, EGCG treatment in cell culture models led to reduced genomic 5meC levels and promoter hypomethylation of selected candidate genes [129-131, 133-136] (summarized in Supplementary Table **3**). This was generally associate with mRNA re-expression of these genes, with the exception of *hTERT*. *hTERT* is a catalytic subunit of the enzyme telomerase, which is upregulated in cancer cells. *hTERT* transcription is repressed through binding of the repressor *E2F* to its promoter region. In tumor cells, methylation at the *E2F* binding site prevents *E2F* binding. EGCG treatment of MCF-7 breast cancer cells resulted in demethylation of selected CpG sites, enhanced *E2F* binding measured by chromatin immunoprecipitation (ChIP), and reduced *hTERT* mRNA levels [134]. Kato *et al*. investigated the effect of EGCG on the methylation status of *RECK* (reversion-inducing cysteine-rich protein with Kazal motifs), a novel tumor suppressor gene with *matrix-metalloprotease* (*MMP*)-inhibitory activities. Treatment of oral squamous carcinoma cell lines with EGCG led to partial promoter demethylation and mRNA reexpression of *RECK*, which was associated with reduced *MMP* levels and activity and inhibition of invasive potential through collagen matrices [135]. Also, EGCG potently reduced promoter methylation of *WIF-1* (*Wnt inhibitory factor1*) in lung cancer cell lines, resulting in down-regulation of Wnt signaling [136]. *WIF-1* is a *Wnt* antagonist that inhibits *Wnt* signaling through direct binding to *Wnt*. *Wnt* signaling plays an important role during embryonic tissue development and tissue homeostasis in adults, and aberrant *Wnt* signaling has been implicated in cancer development in various organs, including colon, skin, liver, ovary, breast and lung [137]. Two studies by Chuang *et al*. [138] and Stresemann *et al.* [139] could not confirm demethylating activity and *p16* mRNA reexpression after EGCG treatment of T24 bladder cancer cells, HT 29 and HCT116 colon cancer cells and PC3 prostate cancer cells

and the TK6 B-lymphoblastoid cell line. Reasons for this discrepancy are presently not known.

At present, data is too limited to conclude whether EGCG and polyphenol-mediated effects on DNA methylation are due to direct *DNMT* inhibition or might additionally involve downregulation of *DNMT* expression. For example, EGCG treatment did not reduce *DNMT-1, -3a, 3b* and *methyl-CpG-binding domain protein 2* (*MBD2*) mRNA levels in KSYE 510 esophageal cells [129]. However, treatment of LNCaP cells with GTP for 2 weeks reduced *DNMT* mRNA and protein expression [131]. Similarly, Fini *et al*. described the effect of an apple polyphenol extract containing a mixture of at least 6 polyphenols on promoter methylation of the DNA repair protein *hMLH1* and two cell cycle regulatory proteins $p14^{ARF}$ and $p16^{INK4A}$ in three colon cancer cell lines. Promoter demethylation was associated with mRNA or protein reactivation of the investigated genes. The effects on DNA methylation were explained by a reduction in *DNMT1* and *-3b* protein levels, although mRNA levels were not reduced [140]. This effect on DNA methylation may contribute to the cancer preventive efficacy of apple polyphenols (reviewed in [141]).

The effects of GTP on DNA methylation were also monitored in two mouse models. Volate *et al*. treated normal B6 and $APC^{Min/+}$ mice prone to develop multiple adenomas in the small intestine with the colon carcinogen azoxymethane. In $APC^{Min/+}$ mice, intervention with GTP (0.6% in drinking water) for 4-8 weeks significantly reduced newly formed small adenomas in the small intestine by 28% and colonic tumor formation by 50%. The *retinoic X receptor* α (*RXR*α) was selectively down-regulated early during AOM-induced carcinogenesis both in $APC^{Min/+}$ and wild type B6 mice due to promoter methylation. GTP intervention reduced promoter methylation and led to an upregulation of *RXR*α mRNA in mucosal preparations. These findings could be relevant for the prevention of human colon carcinogenesis, since *RXR*α expression was also reduced in human colon adenocarcinoma [142]. Morey Kinney *et al.* investigated the potential of GTP to inhibit DNA methylation in the 'TRansgenic Adenocarcinoma of Mouse Prostate' (TRAMP) model *in vivo*. In this study, GTP intervention did neither affected global me5C levels, locus-specific or genome-wide DNA methylation, nor did it reduce the progression of prostatic tumor formation. This is in contrast with earlier studies demonstrating potent effects of GTP intervention on prostate cancer development in the same animal model [143-145].

Only very few epidemiological or human intervention studies with chemopreventive agents have attempted to investigate effects on DNA methylation. Yuasa *et al.* measured promoter methylation of six tumor-related candidate genes, *i.e.* the homeobox transcription factor *CDX2*, bone morphogenetic protein *BMP2*, cyclin-dependent kinase inhibitor $p16^{INK4A}$, calcium channel-related *CACNA2D3*, transcription factor *GATA-5* and estrogen receptor *ER*, in primary tumor samples of 106 gastric cancer patients by methylation specific PRC [146]. *CDX2* and *BMP2* promoter methylation was significantly more frequent in patients with low green tea consumption than in those with $\geq$ 7 cups of green tea per day. Also, more physical activity was correlated with a lower methylation frequency of *CACNA2D3*.

Interestingly, the effects of EGCG on the epigenome are not limited to DNA methylation. A recent study suggests that EGCG reduces skin tumor cell survival by influencing *polycomb group* (*PcG*)-mediated histone modifications [147]. The *polycomb group* (*PcG*) proteins, including *BMI-1* and *EZH2,* are epigenetic regulators of gene expression by increasing H3K27 methylation and reducing acetylation, leading to a repressed chromatin conformation and enhanced cell survival. EGCG treatment of SCC-13 skin cancer cells reduced levels of *BMI-1* and *EZH2*, consistent with reduced cell survival and a reduction in H3K27me3 levels mediated by *EZH2*. EGCG treatment also modulated the expression of cell cycle regulating proteins and induced apoptosis mediated by activation of *caspase 9, 8* and *3* and *Bcl-2* family proteins. The inhibitory effects of EGCG on *BMI-1* expression were corroborated by overexpression of *BMI-1*. These findings demonstrate that EGCG suppresses the survival of SCC-13 cells through a mechanism depending on inhibition of *BMI-1*-driven H3K27 methylation.

Tsang *et al.* recently reported that EGCG also affects the expression of miRNAs, adding to its spectrum of chemopreventive mechanisms related to epigenetic gene regulation. Using a miRNA microarray approach, they found that EGCG treatment up-regulated the expressions of 13 miRNAs and down-regulated expression of 48 miRNAs in HepG2 human hepatocellular carcinoma cells [148]. One of the up-regulated miRNAs was *miR-16*, which was shown to influence the anti-apoptotic protein *Bcl-2*. Consequently, EGCG treatment down-regulated *Bcl-2* and induced apoptosis in HepG2 cells. Transfection with *anti-miR-16* inhibitor confirmed the role of *miR-16* in downregulation of *Bcl-2* and apoptosis-induction by EGCG.

Recent evidence is accumulating that the expression of miRNAs itself is underlying epigenetic control *via* DNA methylation and chromatin modifications [149]. Therefore, the potential impact of preventive agents such as EGCG on the epigenome might be more complex than currently anticipated and requires further thorough investigation.

4.4.2. Genistein and Soy Isoflavones

Isoflavones represent a subgroup of the flavonoid family and are characterized by phytoestrogenic properties. Genistein is the major isoflavone derived from soy. Epidemiological evidence indicates an inverse correlation between a traditional low-fat, soy-rich Asian diet and the risk to develop breast and prostate cancer [150, 151]. This is mainly attributed to the hormonal activity of soy isoflavones mediated by estrogen receptor binding. In addition, genistein acts *via* multiple mechanisms and affects carcinogen bioactivation, cell-signaling, cell cycle regulation, angiogenesis, oxidative stress, and inflammation [72, 152]. Chemopreventive activity of genistein and soy isoflavones has been demonstrated in animal models for ovarian, skin, stomach, and colon cancer, with most studies related to the prevention of prostate and breast cancer [153]. Recent rodent breast cancer studies have indicated growth-promoting activity of genistein for hormone-dependent breast cancer; these data have raised concerns on the safety of genistein for human application [154]. Genistein is currently undergoing clinical

testing for treating prostate, bladder, and kidney cancer, and for prevention of breast and endometrial cancer [155].

Both epidemiological as well as animal studies consistently indicate that exposure to soy products during prepuberty provides protection against breast cancer later in life [156]. These results point to an epigenetic reprogramming by soy isoflavones during early development of the mammary gland [157]. Various hormone receptors including the *estrogen receptors* α and β regulate gene expression through ligand-dependent recruitment of co-activators (*HATs*) or co-repressors (*HDACs*) [158]. In line with this, Hong *et al*. reported that genistein, daidzein and the daidzein metabolite equol stimulated *ER*β-mediated histone acetylation and co-activator activity of *ER* [159]. Additionally, genistein caused upregulation of histone acetyl transferases (*HATs*) in prostate cancer cell lines. This resulted in hyperacetylation of histones H3 and H4, increased association of acetylated H3K4 with the transcription start sites of cell-cycle regulators *p21* and *p16,* and subsequent re-expression [160]. Kikuno *et al.* were interested whether the suppressing effects of genistein on *AKT* signaling might be mediated *via* epigenetic mechanisms. Tumor suppressor *PTEN* frequently lost in prostate cancer is an upstream regulator of *AKT*. Downstream signaling is indirectly mediated *via* transcription factors such as *NF-*κ*B* and *FOXO*. The authors showed that genistein treatment of LNCaP and PC-3 prostate cancer cell lines led to re-expression of *PTEN* and consequential inactivation of *AKT*, resulting in induction of *p53* and *FOXO3a*. Also, genistein treatment upregulated the endogenous *NF-*κ*B* inhibitor *CYLD*, resulting in decreased constitutive *NF-*κ*B* activity. Promoter regions of all of these factors were unmethylated in the investigated cell lines. Rather, re-expression involved H3K9 demethylation (*PTEN* and *CYLD*) and increased H3K9 acetylation (*PTEN, CYLD, p53, FOXO3a*). H3K9 hyperacetylation was shown to be mediated by reduced expression and nuclear localization of the class III deacetylase *SIRT1*. These findings underline the importance of epigenetic mechanisms for the inhibition of *AKT* and *NF-*κ*B* signaling by genistein [161].

Androgen receptor (*AR*) signaling provides the most important growth stimulus in hormone-dependent prostate cancer. *HDAC6* was shown to deacetylate and activate non-histone proteins, including the *AR* chaperone *heat shock protein 90* (*HSP90*). Genistein treatment of LNCaP cells lowered *AR* levels through enhanced proteasomal degradation. This was associated with hyperacetylation of *HSP90*, thereby dissociating the interaction between *HSP90* and *AR*. *HSP90* hyperacetylation was due to reduced levels of *HDAC6*, mediated by anti-estrogenic effects of genistein. The effects of *HDAC6* downregulation by genistein on *AR* were mimicked by *HDAC6* siRNA. These data indicate that prostate cancer preventive potential of genistein may also be mediated through modulating the complex of *HDAC6* with *HSP90* and *AR* [162].

Several *in vitro* investigations demonstrate that genistein is additionally effective in reducing promoter methylation. Genistein treatment of esophageal, prostate and colon cancer cell lines stimulated re-expression of $p16^{INK}$, retinoic acid receptor *RAR*β*2* and the repair protein *MGMT* [125, 163]. On the other hand, King-Batoon *et al*. reported a lack of *RAR*β*2* demethylation in MCF-7 and MDA-MB468 breast cancer cell lines, whereas *GSTP1* mRNA re-expression cells was associated with reduced promoter methylation in MDA-MB468 and also in three prostate cancer cell lines [164, 165]. Genistein treatment of MCF10AT benign breast cells and MCF-7 breast cancer cells decreased the expression of the major DNA methyltransferases *DNMT 1*, *3a* and *3b*. Concomitantly, genistein treatment inhibited human telomerase reverse transcriptase (*hTERT*) transcription by increasing the binding of the repressor *E2F-1* to the *hTERT* core promoter. This was facilitated by site-specific hypomethylation of the *E2F-1* binding site [166]. Genistein intervention in three renal and prostate cancer cell lines resulted in promoter demethylation and mRNA reexpression of tumor suppressor *BTG3*. *BTG3* is a negative regulator of *Scr*-signaling and inhibits *E2F*-1. Reactivation was due to inhibition of *DNMT* activity and partly protein expression. Genistein also inhibited *HDAC* activity and increased *HAT* activity, resulting in increased binding of acetylated and methylated histones to the *BGT* promoter indicating increased transcription [167, 168].

Parker *et al.* addressed the question whether genistein may modulate miRNA expression in a pair of ovarian cancer cell lines established from one patient during progression of disease [169]. Using a miRNA profiling approach, they identified 53 out of 467 analyzed miRNA with differential expression between both cell lines. Genistein treatment affected expression of 18 and 8 miRNA in both cell lines, respectively. This study did not include further validation or investigation of potential targets. Pancreatic cancer is one of the most common causes of cancer death. This is partly due to development of drug-resistance, and the acquisition of epithelial-to-mesenchymal transition (EMT). Treatment of gemicitabine-resistant pancreatic cancer cells with an isoflavone mixture containing genistein resulted in the re-expression of *miR-200* [170]. This was associated with a change in morphology to a more epithelial-like phenotype, which could indicate reversal of the EMT. Weak down-regulation of the mesenchymal markers *ZEB1* and *vimentin* mRNA pointed in the same direction. Recently, genistein was shown to downregulate the expression of members of the *minichromosome maintenance* (*MCM*) gene family in prostate cancer cell lines [171]. *MCM* genes play an important role in DNA replication during cell division and are often upregulated in cancer cells. One of these, *MCM2,* was identified as a target of *miR-1296,* which is downregulated in prostate tumor tissue compared with samples of benign prostatic hyperplasia (BPH). Overexpression of *miR-1296* by transfection reduced *MCM2* mRNA expression and resulted in a strong cell cycle arrest in S-phase. Similarly, genistein dose-dependently induced *miR-1296* up to 5-fold. Therefore, antiproliferative activity of genistein may be mediated *via* a miRNA-mediated effect on cell cycle progression. In another study, Sun *et al.* demonstrated that genistein-treatment reduced proliferation of uveal melanoma cells *in vitro* and in a xenograft model. *MiR-27a* expression was reduced by genistein intervention, leading to increased expression of its target gene *ZBTB10* [172].

In rodent models, genistein or isoflavone exposure modulated promoter methylation of selected genes, e.g. several novel genes in murine prostate [173], the *IAP* murine retrotransposon in viable yellow agouti mice [174], and *skeletal* α*-actin* in murine liver [175]. It also led to an

overexpression of *Nucleosomal binding protein 1* (*Nsbp1*) in mouse uteri due to promoter hypomethylation [176]. Notably, Qin *et al.* recently reported dose-dependent changes in *RARβ2* and *CCND2* promoter methylation in mammary tissue after a 4-week human intervention in healthy premenopausal women [177]. So far, none of these studies investigated the direct relation between modulation of epigenetic mechanisms by genistein and chemopreventive potential *in vivo*.

4.4.3. Parthenolide

The sesquiterpene lactone parthenolide was identified as the bioactive principal of feverfew (*Tanacetum parthenium* (L.) Sch. Bip.), a traditional medicinal herb for the treatment of fever, migraine and arthritis [178]. Parthenolide is a potent inhibitor of transcription factor *NF-κB* (nuclear factor κB) and *STAT* (*signal transducer and activator of transcription*) signaling pathways and induces apoptosis in cancer cells [179].

Inhibition of *NF-κB* activation is partly mediated by direct interaction of parthenolide with a cysteine moiety in the *p65* subunit. This is attributed to its electrophilic α-methylene-γ-lactone ring and exocyclic double bond, which represent Michael acceptor functionalities (α,β-unsaturated carbonyl moiety) with thiol reactivity and allow direct binding to nucleophilic sites of biological molecules. Since *DNMT* contains an essential cysteine in its catalytic pocket, Liu *et al*. argued that parthenolide might inhibit *DNMT* activity and DNA methylation [178]. *In silico DNMT* homology modeling and docking studies with parthenolide, and subsequent *in vitro DNMT* activity testing identified parthenolide as a novel inhibitor of methyltransferase activity, with IC_{50} value of 5.0 μM for bacterial *Sss1*, and 3.5 μM for human *DNMT1*. Comparison with a parthenolide analog lacking the exocyclic double bond, which was shown to be devoid of any inhibitory activity, indicated the importance of this functionality for the observed *DNMT* inactivation. Liu *et al.* further demonstrated dose-dependent reduction of *DNMT* expression in MV4-11 and Kasumi human leukemia cell lines, which might be related to parthenolide-mediated cell cycle arrest, apoptosis induction, and prevention of transcription factor *Sp1* binding to the *DNMT1* promoter. Inhibition of *DNMT* activity and expression was associated with global DNA hypomethylation, both *in vitro* and *in vivo* in MV4-11 xenografts, and reduced tumor growth [178].

4.4.4. Curcumin

Curcumin (diferuloyl methane) is a yellow pigment and the major active ingredient of turmeric (*Curcuma longa)*. It belongs to the best investigated cancer chemopreventive agents, and its activities have been summarized in various previous reviews (for example [180-182]). Curcumin is well tolerated and non-toxic, even at elevated concentrations. It has been shown to suppress tumor growth through multiple signaling pathways, particularly *NF-κB* signaling, and inhibit cell proliferation, invasion, metastasis, and angiogenesis *in vitro* and in rodent models for breast, prostate, colon, esophagus, stomach, liver, lung, oral and skin cancers. Several clinical trails in patients with inflammatory diseases and cancer have shown promising first results [181]. Current research efforts also focus on potential synergistic effects of co-treatment with curcumin and cancer-therapeutic agents, and on reversal of drug resistance [183].

With respect to epigenetic mechanisms, histone acetyl transferases (*HATs*) were first postulated as potential targets of curcumin. Balasubramanyam *et al*. identified curcumin as a specific inhibitor of *p300/CBP in vitro* and in cell culture, whereas *PCAF*, *HDAC* and histone methyltransferase activities were not inhibited by curcumin even at elevated concentrations [184]. The authors suggest that curcumin might modify the structure of *p300*, thereby preventing binding of histones or cofactor acetyl-CoA. Curcumin also inhibited acetylation of *p53*, which represents a non-histone target of *p300/CBP*. Also, it specifically repressed the *p300/CBP HAT* activity-dependent transcriptional activation from chromatin, but not a DNA template. In another study, exposure of human hepatoma cells to curcumin led to a significant decrease in histone acetylation. Curcumin treatment significantly inhibited *HAT* activity both *in vitro* and treated cells [185]. Incubation with curcumin resulted in a comparable inhibition of histone acetylation in the absence or presence of TSA and showed no effect on the *in vitro* activity of *HDAC*. Also, a dominant negative form of *p300* could block the inhibition of curcumin on histone acetylation. Therefore, the authors suggested that *HAT*, but not *HDAC*, is involved in curcumin-induced histone hypoacetylation. Interestingly, histone hypoacetylation was associated with increased production of reactive oxygen species (ROS), and co-treatment with the antioxidant enzymes superoxide dismutase and catalase could inactivate both ROS and histone acetylation. The mechanism how these effects could be related was not resolved. Chen *et al*. investigated the effect of curcumin on *NF-κB* and histone modifying proteins in Raji cells [186]. Curcumin treatment led to a significant and dose-dependent decreases in the amounts *HDAC1* and *p300* protein and mRNA levels. Reduction was prevented by co-treatment with MG-132, an inhibitor of the 26S proteasome. Interestingly, *p300/CBP HATs* can enhance *NF-κB* transcriptional activity as co-activators by acetylating both *NF-κB/p65* and surrounding histones. Direct inhibition and down-regulation of *p300/CBP* could therefore contribute to the well-known inhibition of *NF-κB* by curcumin [187].

Similar to parthenolide, curcumin harbors a Michael acceptor functionality in its chemical structure. This prompted Liu *et al.* to perform *in silico DNMT* docking studies with curcumin and related analogs [188]. These data indicate that curcumin may compete with cofactor SAM for binding to the catalytic pocket of *DNMT*. Inhibition of methyltransferase activity was confirmed with bacterial *Sss*1, and an IC_{50} value of 30 nM was determined [188]. Treatment of MV4-11 human leukemia cells with curcumin led to global hypomethylation, but sequence-specific demethylation at promoter regions of epigenetically silenced genes has not been demonstrated yet.

Several recent studies report that curcumin targets miRNA-regulated gene expression. Sun *et al*. investigated the effect of curcumin on expression profiles of miRNAs in BxPC-3 human pancreatic cancer cells using a custom miRNA microarray. Treatment with curcumin resulted in the significant upregulation of 11 miRNAs and downregulation of 18 miRNAs. Two miRNAs, *miR-22* and *miR-199a** were selected for further conformation by quantitative RT-PCR.

Predicted target genes of *miR-22* include *ERα* and transcription factor *Sp1*, respectively. Curcumin treatment indeed resulted in the downregulation of *ERα* and *Sp1* protein expression. Similar results were observed when BxPC-3 cells were transfected with sense *miR-22* oligonucleotides. Expression of *ERα* and *Sp1* was reversed by transfection with antisense *miR-22* oligonucleotides, confirming them as functional targets of *miR-22* [189]. In another study with pancreatic cancer cells, curcumin and CDF, a fluoro-derivative with improved bioavailability, were initially tested for potential to restore gemcitabine sensitivity by single and combination treatment experiments [190]. Beside cell growth inhibition, apoptosis induction, and modulation in the expression of important apoptosis-regulating proteins, both compounds normalized the expression of *miR-200* and oncogenic *miR-21*, which were deregulated in gemcitabine-resistant cells relative to -sensitive cells. Downregulation of *miR-21* by curcumin resulted in the induction of the tumor suppressor gene *PTEN*, which is often lost in malignant tumors. Studies on apoptosis induction by curcumin in multidrug-resistant lung cancer cells also include miRNA-related mechanisms of apoptosis induction [191]. Out of 342 human miRNA analyzed by miRNA microarray analysis, curcumin-treatment resulted in a >2.5-fold up-regulation of four miRNAs, and down-regulation of *miR-186** and *miR-136* known to be up-regulated in lung cancer. Overexpression of *miR-186** by transfection with a mimic oligonucleotide confirmed its potential oncogenic and anti-apoptotic role and attenuated apoptosis-induction by curcumin. These data indicate that miRNA-mediated mechanisms contribute to the chemopreventive potential of curcumin, and that curcumin targets the epigenome by multiple mechanisms (recently reviewed in [79]).

4.4.5. Ellagitannin

Ellagitannins are a family of bioactive oligomeric or polymeric polyphenols that occur in high concentrations in fruits and nuts, such as raspberries, strawberries, almonds, and walnuts. They are polyesters of a sugar moiety and ellagic acid and release ellagic acid upon hydrolysis (hydrolysable tannins). They form urolithins after further metabolism by the gut microflora. Ellagitannins are characterized by prominent anti-oxidant activity and radical scavenging, antiviral, antimicrobial, antimutagenic, anti-inflammatory, anti-tumor promoting, immunomodulatory, anti-proliferative and apoptosis-inducing properties. Selected structures have been shown to reduce tumor growth *in vivo* [192].

A particular ellagitannin (BJA3121, 1,3-di-O-galloyl-4,6-(s)-hexahydroxydiphenoyl-β-D-glucopyranopside) was isolated from *Balanophora japonica*, a plant from Japan and China used in Traditional Chinese Medicine, and was shown to possess anti-proliferative activity in human liver cancer cells. Wen *et al.* investigated whether cell growth inhibition was related to altered expression of miRNAs. Using a microarray approach, they demonstrated that BJA3121 treatment resulted in the up-regulation of 17 miRNAs and down-regulation of 8 miRNAs in HepG2 cells after 6 h of incubation. Expression of seven miRNAs selected for validation (upregulated: *let-7e*, *miR-370, miR-373*, miR-526b*; down-regulated: *let-7a, let-7c, let-7d*) was modulated by BJA3121 in a dose- and time-dependent manner, as confirmed by qRT-PCR analysis. Several of these miRNAs have been shown to respond to treatment with anti-cancer drugs, for example 5-aza-2'-deoxycytosine. Confirmed targets of miRNAs with altered expression after BJA3121 treatment are mainly involved in regulation of cell differentiation and proliferation. The precise mechanisms for the altered miRNA expression has not been investigated yet [193].

4.4.6. Indole-3-carbinol (I3C), Diindolylmethane (DIM)

Vegetables of the *Cruciferae* family, in particular those of the *Brassica* genus (broccoli, cabbage, cauliflower, radish, mustard etc.) have received much attention because of their cancer preventive and cancer reducing activities *in vitro* and *in vivo* (comprehensive review in [194]). Thioglucoside conjugates, namely glucosinolates, are responsible for the chemopreventive activity of cruciferous vegetables. Through catalytic mediation of myrosinase (β-thioglucosidase), which is released on physical damage of the plant cell (e.g. during cutting or chewing), the glucosinolates are hydrolyzed, releasing the corresponding isothiocyanates (ITC, see below). Indole-3-carbinol (IC3) is the main hydrolysis product of the glucosinolate glucobrassicin. I3C is especially known for its protective effect on the carcinogenesis of reproductive organs [195]. Under low pH conditions, as in the stomach, condensation of I3C results in the formation of 3,3'-diindolylmethane (DIM) as the major condensation product. Both compounds suppress cell proliferation and induce apoptosis in breast, prostate, cervical and colon cancer cell lines ([196] and references cited therein). Also, they have been shown to modulate nuclear receptor- and kinase-mediated signaling pathways, induce ER stress, and possess anti-angiogenic activities [195].

Both I3C and DIM have recently been tested for potential to modulate the expression of miRNAs. Li *et al.* addressed the question whether DIM might affect epithelial to mesenchymal transition (EMT) in gemcitabine-resistant pancreatic cancer cells (as described in 4.4.2 above). miRNA profiling identified 28 miRNAs that were differentially expressed after treatment with 25 μM DIM for 48 h. Up-regulation of members of the *let-7* (*let-7b, let-7e*) and miR-200 families (*miR-200b*, *miR-200c*) was confirmed by quantitative miRNA RT-PCR. After cultivation of pancreatic cancer cells with low-dose DIM for 3 weeks, the epithelial marker *E-cadherin* was up-regulated at the mRNA level, whereas protein or mRNA levels of the mesenchymal markers *ZEB1* and *vimentin* were downregulated [170], indicating DIM-induced reversal of the EMT phenotype. In a second study, the same group investigated whether DIM may influence invasion capacity of pancreatic cells *via* a miRNA-regulated mechanism [197]. Pancreatic cancer cells express lower levels of *miR-146* than normal pancreatic duct epithelial cells. Re-expression of *miR-146* by transfection reduced the expression of *EGFR* (*epidermal growth factor receptor*), and members of the *NF-κB* signaling pathway, including *IRAK-1* (*interleukin 1 receptor-associated kinase 1*), *IκB*, and *NF-κB*. Also, the *metastasis-associated protein 2* (*MTA-2*) was downregulated, and the invasive capacity of the cells was reduced. Interestingly, all these effects could be mimicked *via* upregulation of *miR-146* by incubation with 25 μM DIM [197].

In a study by Izzotti *et al*. treatment of rats with environmental cigarette smoke (ECS) for 4 weeks resulted in extensive downregulation of miRNA expression in lung tissue [198]. Pre-treatment of the rats for 3 days with five chemopreventive agents, including I3C, phenethyl isothiocyanate (PEITC, described below), n-acetylcysteine, oltipraz, and 5,6-benzoflavone alone or in combination, attenuated the changes on miRNA expression. I3C strongly counter-regulated the expression of 7 out of 25 miRNAs down-regulated by ECS, including miRNAs involved in *p53* functions (*miR-34b*), *TGF-β* expression (*miR-26a*), *ERBB2* activation (*miR-125a-prec*), and angiogenesis (*miR-10a*). Future studies have to confirm whether these direct or indirect effects on miRNA by I3C and DIM correlate with cancer prevention *in vivo*.

4.4.7. Mahanine and Mahanine Derivative

Mahanine is a carbazole alkaloid found in some Asian vegetables. It has been shown to exhibit anti-mutagenicity against heterocyclic amines, anti-microbial activity against *Gram-positive* bacteria, anti-inflammatory effects, cytotoxicity and apoptosis induction in various tumor cell lines including prostate cancer cells (summarized in [199]). Jagadeesh *et al*. addressed the question whether cell growth inhibition in LNCaP and PC-3 prostate cancer cells might be related to the reactivation of a tumor suppressor gene. Mahanine treatment reduced *DNMT* activity in prostate cancer cells, and reactivated *RASSF1A* mRNA expression in several human tumor cell lines. *RASSF1A* expression was associated with reduced *cyclin D1* protein expression, whereas the expression of other cyclins was not affected. In continuation of this study, a series of mahanine derivatives were synthesized and tested for effects on PC-3 proliferation, *RASSF1A* and *cyclin D1* mRNA expression, and *DNMT* activity [200]. One compound, derivative 9, was equally effective as mahanine in inhibiting PC-3 cell proliferation. Also, it similarly decreased DNA synthesis, inhibited *DNMT* activity more potently than mahanine, reactivated *RASSF1A* mRNA expression, and downregulated *cyclin D1*, which might be relevant for the observed cell growth inhibition. Compound 9 harbors a dansyl moiety, allowing fluorescent detection. Interestingly, the compound was localized exclusively in the cytoplasm of PC-3 cells, and was able to sequester *DNMT3b*, but not *DNMT3a* in the cytoplasm. Depletion of *DNMT3b* has been shown previously to cause *RASSF1A* re-activation, cell growth inhibition and apoptosis induction in cancer cell lines, but not in normal human mammary and foreskin cells [201]. Compound 9 was also tested *in vivo* and did not cause oral toxicity in Balb/c mice at concentrations up to 550 mg/kg. It reduced growth of PC-3 xenografts by 40% when applied at 10 mg/kg b.w. every other day for 4 weeks. The influence of epigenetic mechanisms for tumor growth inhibition was however not further investigated.

4.4.8. 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), which grows in arid regions in Northern Mexico and Southwestern United States. NDGA has potent anti-oxidant activities and is a known inhibitor of lipoxygenases. Tumor cell growth inhibitory potential *in vitro* and *in vivo* has been associated with inhibition of receptor tyrosine kinases such as *insulin-like growth factor receptor 1* (*IGF-1R*). NDGA also possesses cancer chemopreventive activity in both UVB-induced and chemically-induced models of carcinogenesis for breast, prostate, lung, esophageal, and skin cancer [202, 203]. NDGA is undergoing early clinical trials for prostate cancer treatment, but its use might be limited by hepatotoxicity [203, 204].

Early reports from China gave first indication that NDGA inhibits *DNMT* activity and reduces global methylation in malignant glioma cells (cited in [205]). Based on these results, Cui *et al*. investigated the influence of NDGA on epigenetically silenced *E-cadherin* expression in human breast and colon cancer cell lines. Consistently, intervention with NDGA for up to 7 days reduced *E-cadherin* promoter hypermethylation, accompanied by increased mRNA and protein expression. In MDA-MB-435 xenografts, NDGA treatment also resulted in increased *E-cadherin* protein expression and reduced tumor growth [205, 206]. *p16* reactivation in RKO and T47D cells was associated with reduced *cyclin D1* expression and *RB* phosphorylation, G_1 cell cycle arrest, and increased senescence [205]. In contrast, in HepG2 cells NDGA treatment did not reduce *LINE-1* methylation as a sign of global demethylation [207].

4.4.9. Lycopene

Carotenoids are a class of about 600 naturally occurring tetraterpenoids. The carotenoid lycopene is mainly contained in tomatoes and tomato products. Epidemiological studies indicate that regular consumption of tomato products may be related to reduced prostate cancer risk. Lycopene was found to reduce tumor growth in animal models for prostate, breast, and lung cancer, whereas it was ineffective in preventing colon, kidney and liver cancer (reviewed in [208]). Lycopene is a potent antioxidant and $^1O_2$ quencher, and has been shown to reduce oxidative DNA damage. Interestingly, it accumulates in the nucleus in prostate cells, which might explain its preferential action in prostate cancer prevention. In cell culture, lycopene activates drug metabolism and inhibits cell proliferation by induction of apoptosis and cell cycle arrest. At higher concentrations, it has anti-invasive and anti-metastatic properties. Lycopene is undergoing Phase 2 clinical testing. In five studies applying lycopene to subjects with prostatic intraepithelial neoplasia (PIN) and prostate cancer patients for 3 weeks – 16 months, intervention reduced oxidative DNA damage in leukocytes, but did not effect PSA level or progression of disease. Nevertheless, lycopene is considered as a promising agent for prostate cancer prevention.

King-Batoon *et al*. investigated the effects of lycopene on DNA methylation in breast (cancer) cells. A single application of 2 μM lycopene reactivated *GSTP1* mRNA expression, associated with reduced promoter methylation in MDA-MB-468 cells. *RARβ* and *HIN1* promoter methylation was reduced in immortalized MCF10A human breast cells, but not in MCF-7 breast cancer cells [164]. The mechanism of DNA demethylating activity and further functional consequences have not been further investigated.

4.5. Sulfur-Containing Compounds

4.5.1. Sulforaphane and PEITC

Sulforaphane and PEITC (phenethyl isothiocyanate) are dietary isothiocyanates derived from *Cruciferous* vegetables such as broccoli and water cress, respectively. Both SFN and PEITC target multiple mechanisms relevant for chemoprevention (reviewed in [194, 209-213]): they potently modulate carcinogen metabolism by inhibition of Phase I and induction of Phase II enzymes, block *NF-κB* and hormone-receptor signaling, inhibit cell proliferation by induction of apoptosis and cell cycle arrest, and were further shown to induce angiogenesis and autophagy [214-217]. SFN intervention has been shown to prevent carcinogen- or genetically-induced skin, lung, breast, prostate, pancreas, stomach and colon cancer in rodent models, and to inhibit prostate and pancreatic xenograft growth [210]. PEITC has been extensively studied in carcinogen-induced experimental models for lung cancer and a series of other tumor types [212], and effectively inhibits tumor growth in xenograft models [217, 218]. First clinical trials with both compounds have been initialized [219].

Sulforaphane (SFN)

Potential of isothiocyanates to target the epigenome was first described in 2004 (review in [220]). In a seminal study, Myzak *et al.* postulated that SFN might possess *HDAC*-inhibitory activity, based on the observation that SFN treatment caused *p21* upregulation and cell cycle arrest, similar to the activities of butyrate. SFN did not directly inhibit *HDAC* activity in cell-free systems *in vitro*, but *in silico* modeling predicted that SFN-Cys, a SFN metabolite, might fit into the catalytic pocket of *HDAC*s. Consistently, after incubation of HeLa cells with SFN, the media contained a metabolite able to inhibit *HDAC* enzymatic activity in a cell-free system [221]. Further studies confirmed the *HDAC* inhibitory activity of SFN in human colon, prostate and breast cancer cells [221-223]. SFN treatment increased global acetylation of histone H3 and H4 in human prostate cancer cells, accompanied by locus-specific hyperacetylation of H3, H4 or both at the *p21* promoter. A recent studies by Gibbs *et al.* provides a more detailed molecular basis for the prostate chemopreventive potential of SFN [224]. SFN treatment of LNCaP cells induced rapid hyperacetylation of *HSP90* through inhibition of *HDAC6* activity, resulting in dissociation of the *AR*. As a consequence, *AR* levels were reduced through proteasomal degradation, leading to lowered *AR* occupancy at target genes and decreased expression of *PSA* and *TMPRSS2-ERG*. The effects of *HDAC6* inhibition by SFN on *AR* were restored by overexpression of *HDAC6* and mimicked by *HDAC6* siRNA or treatment with TSA. Therefore, similar to genistein [162], SFN may act as a prostate cancer preventive agent by affecting the complex of *HSP90-AR* through *HDAC6* inhibition [224].

Only recently, Meeran *et al.* additionally identified SFN as a DNA demethylating agent. Cancer cells are characterized by high telomerase activity and elevated expression of *hTERT*, the catalytic subunit of telomerase. SFN treatment of breast cancer cell lines inhibited telomerase activity and repressed *hTERT* mRNA expression. This was associated with downregulation of *DNMT1* and *DNMT3a* protein expression and a significant reduction in *hTERT* methylation at CpG sites in exon 1, especially at the binding region of *hTERT* repressor transcription factor *CTCF*. ChIP analysis of the *hTERT* promoter showed increased levels of ac-H3, H3K9ac and ac-H4, whereas the H3K9me3 and H3K27me3 inactive chromatin markers were decreased. SFN-induced hyperacetylation facilitated the binding of *MAD1* and *CTCF* and decreased binding of *c-Myc*. Knockdown of *CTCF* restored *hTERT* expression and decreased the apoptosis-inducing potential of SFN. In addition, SFN-treatment inhibited *HDAC* activity and may modulated histone methylation by increased expression of the histone demethylase *RBP2* [225, 226].

Treatment of wild-type (wt) and $APC^{Min/+}$ mice underline the relevance of epigenetic mechanisms contributing to the chemopreventive activity of SFN. In wild-type mice, a single dose of SFN reduced *HDAC* activity and transiently increased ac-H3 and ac-H4 levels in colonic mucosa [227]. Similar effects were observed in ileum, colon, prostate and peripheral blood mononuclear cells (PBMC) when SFN was applied over 10 weeks. SFN treatment reduced tumor multiplicity in $APC^{Min/+}$. In tumor samples, ac-H3 levels correlated with increased ac-H3 occupancy at the *p21* and *Bax* promoters, and induced expression of proapoptotic *Bax* [227]. SFN reduced growth of androgen-independent human prostate cancer cells in a xenograft model, upregulated the expression of *Bax,* and increased global histone acetylation prostate tissue and in xenografts [228]. These findings in support a role for SFN as an HDAC inhibitor *in vivo*, with evidence for decreased HDAC activity in various tissues, increased global acetylation, as well as enhanced localization of acetylated histones at specific promoters. These findings may also be relevant for human cancer prevention. In a pilot study, 3 healthy volunteers ingested 68 g of broccoli sprouts as a source of SFN. Strong induction of histone H3 and H4 acetylation in PBMCs coincided with HDAC inhibition at 3 and 6 h, and returned to normal levels by 24 and 48 h [226].

PEITC

Other isothiocyanates were also shown to target epigenetic mechanisms. Wang *et al.* demonstrated that PEITC treatment reactivated *GSTP1* silenced in prostate cancer cells by a dual mechanism involving histone modifications and promoter demethylation [229]. Exposure of LNCaP cells to PEITC significantly enhanced histone acetylation, cell cycle arrest, and *p53*-independent up-regulation of cyclin-dependent kinase inhibitors, including $p21^{WAF1}$ and *p27* [230]. PEITC treatment also significantly reduced *c-Myc* expression, which is known to repress *p21*. The authors argue that inhibition of *HDACs* rather than *c-Myc* downregulation may be the primary mechanism for *p21* activation. In the TRAMP mouse model for prostate cancer, intervention with PEITC significantly reduced prostate tumor formation and lowered *MGMT* promoter methylation in tumor tissue [231].

Results by Izzotti *et al.* indicate that PEITC also modulates the expression of miRNAs induced by smoking. Rats were pretreated with five different chemopreventive agents including PEITC and I3C (as described above) for three days, before the animals were exposed to environmental cigarette smoke (ECS) for 4 weeks. PEITC intervention either alone or in combination with IC3 was among the

treatments with the strongest protective effects. PEITC strongly counter-regulated the expression of 18 out of 25 miRNAs downregulated by ECS. Main functions of miRNAs that were modified by PEITC include stress response (*miR-125b*), *TGF-β* expression (*miR-26a*), *NF-κB* activation (*miR-146-prec*), *Ras* activation (*let-7a, let-7c*, and *miR-192*), cell proliferation (*let-7a, let-7c*, and *miR-222-prec*), apoptosis (*miR-99b*), and angiogenesis (*let-7a, let-7c, miR-123-prec*, and *miR-222-prec*)[232]. So far, no information is available on the mechanisms that contribute to the protection from ECS-induced miRNA downregulation by PEITC. Since PEITC modulates carcinogen metabolism by inhibition of Phase I and induction of Phase II enzymes, part of the effect may be due to altered metabolism and enhanced detoxification and excretion of tobacco smoke carcinogens.

In another study by the same group, the effect of PEITC or the glucocorticoid budesonide on miRNA expression in mouse liver and lung was analyzed after treatment with the compound alone (starting after weaning for 2 weeks), or in combination with exposure to ECS, started directly after birth [233]. Intervention with PEITC alone had little effect on miRNA expression in lung, but resulted in significant >2-fold downregulation of 9 miRNAs and up-regulation of 3 miRNAs in mouse liver. As in the rat study, PEITC protected the lung from ECS-induced miRNA alterations. The effect on miRNA expression in mouse liver was more complex. PEITC treatment altered the expression in comparison with ECS-exposed animals, but did not restore the miRNA expression profile seen in unexposed mice. In comparison to the ECS-treated group, co-treatment significantly up-regulated 12 miRNAs, whereas 11 miRNAs were down-regulated. Both up- and down-regulated miRNAs were associated with functions in stress response, protein repair, cell proliferation, inflammation and others. Some of the observed alterations in murine liver might indicate adverse effects and need to be further investigated.

4.5.2. Phenylhexyl Isothiocyanate (PHI)

PHI is a synthetic isothiocyanate that also potently inhibited lung tumors induced by the tobacco carcinogen NNK, but not by benzo*[a]*pyrene. Conversely, it enhanced carcinogen-induced tumorigenesis in rat esophagus and colon [212]. As SFN and PEITC, PHI was first identified as an HDAC inhibitor, but was also described to reduced *p16* promoter methylation in myeloma cells and to induce cell cycle arrest [234]. HDAC inhibitory potential and chromatin modifications have been described in a series of studies with human prostate and liver cancer, leukemia and myeloma cells. PHI reduced the expression of *HDAC1* protein and inhibited *HDAC1/2* activities in LNCaP and HL-60 cells [235, 236]. In Molt4 leukemia cells, it increased the level of histone acetyl transferase *p300/CBP* [237]. Increased global acetylation of H3 and H4 was seen in all cell lines, as well as in bone marrow of AML patients [238], associated with increased hyperacetylated histones at the *p21* promoter, enhanced *p21* expression, G_0/G_1 cell cycle arrest, and apoptosis induction. Apoptosis was not induced in mononuclear cells from normal peripheral blood and bone marrow. PHI also modified histone methylation, including upregulation of H3K4 methylation and decreased H3K9 methylation [236, 237, 239].

These results demonstrate that in addition to their established chemopreventive mechanisms, epigenetic mechanisms targeted by ITCs may well contribute to their chemopreventive efficacy *in vivo*. It will be of interest to prove the direct correlation between effects on DNA methylation, histone modifications and miRNA and prevention or reduction of tumor growth.

4.5.3. Diallyldisulfide (DADS) and Allyl-Mercaptan (AM)

Diallyl disulfide (DADS) is an organosulfur compound that is frequently found in garlic and other *Allium* species. Epidemiological evidence indicated that regular consumption of *Allium* vegetables is inversely related to the risk to develop stomach and colon cancer [240]. Since gastric carcinogenesis is promoted by infections with *Helicobacter pylori,* protective effects were partly attributed to the broad-range antimicrobial activity of sulfur-containing compounds from *Allium* species [241]. Further chemopreventive mechanisms include induction of carcinogen detoxification, inhibition of DNA adduct formation, free radical scavenging, inhibition of tumor cell proliferation, induction of cell cycle arrest and apoptosis, inhibition of angiogenesis, and suppression of metastasis. These activities offer protection against chemically-induced cancer in animal models, including forestomach, esophagus, colon, mammary gland, and lung cancer, and inhibit the growth of cancer cells in xenograft models (review in [241]).

The garlic compound DADS generates the active metabolite *S*-allylmercaptocysteine (SAMC). Both compounds are further metabolized to allyl mercaptan (AM) and other metabolites (reviewed in [220]). Induction of histone acetylation by DADS and SAMC was first described in murine erythroleukemia cells [242]. Interestingly, in *in vitro HDAC* inhibition experiments, the metabolite AM was identified as a more potent *HDAC* inhibitor than the precursor compounds DADS and SAMC. These results prompted Nian *et al.* to investigate this metabolite in more detail. They predicted direct binding to the *HDAC* active site by *in silico* docking studies and confirmed inhibitory potential in activity assays with cell lysates, purified *HDAC8*, and in cell culture. *HDAC* inhibition led to increased global ac-H3 and ac-H4, enhanced ac-H3 binding to the *p21* promoter, upregulation of *p21*, and cell cycle arrest [243]. Transient histone hyperacetylation was induced by DADS in various cancer cell lines, followed by *p21* upregulation, cell-cycle arrest and induction of differentiation and apoptosis (reviewed in [244]). Histone acetylation was also observed in normal hepatocytes and colonocytes after application of DADS (200 mg/kg b.w.) by intracaecal perfusion or intraperitoneal injection to male rats [245]. Effects on histone acetylation and downstream mechanisms induced by DADS, SAMC and their metabolite AM may be relevant for preventive efficacy. It should however be mentioned that concentrations of DADS used both for *in vitro* as well as for *in vivo* investigations exceeded those that can be achieved by dietary consumption of *Allium* vegetables. Also, direct effects on HDAC activity and histone acetylation were transient. This may indicate that the compounds would have to be regularly consumed to achieve long-term effects *in vivo*.

4.6. Antibiotics

4.6.1. Mithramycin A (MMA)

Mithramycin A (MMA, also known as aureolic acid, plicamycin, antibiotic LA-7017, PA-177) is a tricyclic polyketide produced by soil bacteria. It was first identified as an antibiotic against *Gram-positive* bacteria, but also possess anti-tumor activity. MMA induces cellular differentiation and has been used to treat chronic myeloid leukemia and testicular cancer [246, 247]. Cancer preventive potential has not been investigated yet.

MMA and structurally related compounds have been shown to interact with the DNA minor groove at regions with high GC content in a non-intercalative manner. This was associated with the inhibition of genes with GC-rich regions in their promoter region, preventing the binding of regulatory proteins [246]. GC-rich DNA-binding properties prompted Lin *et al.* to investigate whether MMA might block *DNMT* methylation activity. Low-dose treatment for 14 days resulted in promoter demethylation and mRNA reexpression of two anti-metastatic tumor suppressor genes *SLIT1* and *TIMP3* in CL1-5 lung cancer cells. This was associated with anti-invasion activity *in vitro*. Molecular modeling indicates that MMA fits into the catalytic pocket of *DNMT1*. In addition, *DNMT* protein, but not mRNA expression was reduced by MMA treatment of CL1-5 and A549 cells. The authors propose a triplex complex formed with MMA, *DNMT1* and double-stranded DNA as a potential mechanisms of demethylating activity, which would lead to depletion of *DNMT1* [247].

Bianchi *et al*. investigated miRNA expression during MMA-induced erythroid differentiation in K562 erythroleukemia cells. *miR-210* expression, but not that of *miR-155*, *miR-221* and *miR-222*, was dose-dependently induced, and induction correlated with the degree of differentiation indicated by benzidine staining. *miR-210* induction was also associated with γ-globin expression [248].

4.6.2. Apicidin

Apicidin, a fungal metabolite, is a cyclic tetrapeptide antibiotic with broad spectrum antiparasitic, antiprotozoal and potential antimalarial properties. These antibiotic properties have been related to the inhibition of *HDAC* [249].

Stimulated by anti-proliferative properties of other *HDAC* inhibitors, apicidin was shown to inhibit cell growth of various cancer cell lines at low µg/ml concentrations. Treatment of HeLa human cervical cancer cells led to induction of morphological changes, G_1 cell cycle arrest and accumulation of ac-H4. In addition, apicidin induced expression of *p21* and *gelsolin* involved in cell cycle control and cell morphology, respectively. Induction of *p21* was accompanied by decreased phosphorylation of *Rb* protein, indicating inhibition of cyclin-dependent kinases. In contrast to the dietary HDAC inhibitors described above, the effects of apicidin on cell morphology, expression of *gelsolin*, and *HDAC1* activity appeared to be irreversible [249].

Recently, You *et al.* showed that apicidin treatment selectively reduced *DNMT1* protein expression in HeLa cells and several other cell lines. Apicidin-mediated downregulation of *DNMT1* was independent of *p21* induction and cell cycle arrest. Rather, reduced *DNMT1* expression was partly due to inhibition of transcription. Analyzes of underlying mechanisms by ChIP experiments indicated that apicidin-treatment reduced local H3 and H4 hypoacetylation at the transcription factor E2F binding site in the *DNMT1* transcription initiation region, although global acetylation of H3 and H4 increased. This was accompanied by the recruitment of *pRB* and the replacement of the histone acetyltransferase *PCAF* with *HDAC1*. In addition, apicidin treatment depleted H3K4me3 and enriched repressive marks H3K9me3 and H3K27me3 on nucleosomes associated with the *DNMT1* transcriptional start site. Pre-treatment with the protein synthesis inhibitor cycloheximide blocked the recruitment of *pRB* and *HDAC1* to the E2F binding site, indicating that the transcriptional repression of *DNMT1* by apicidin requires *de novo* protein synthesis. The nature of this essential protein has not been identified yet. Since most cervical cancer cells are infected with human papilloma virus, the authors speculate that apicidin might interfere with the function of viral oncoproteins binding to *p53* and *pRb* [250].

Theses findings indicate that *DNMT1* overexpressed in HeLa cells could be a target for therapy and maybe even prevention of human cervix cancer. Cervical cancer is the second most common cancer in women worldwide. So far, apicidin has not been tested in animal models for chemopreventive activity.

4.7. Pharmacological Agents

4.7.1. Celecoxib and DFMO

Celecoxib belongs to the class of 'Non-Steroidal Anti-Inflammatory Drugs' (NSAIDs). It selectively inhibits *cyclooxygenase 2* (*Cox-2*), a key inflammation enzyme that converts arachidonic acid to prostaglandins. *Cox-2* overexpression is a prominent feature in tumorigenesis, and elevated levels of prostaglandins in tumor tissue block apoptosis and promote cell proliferation, angiogenesis and invasion [251]. Epidemiological evidence indicates that regular use of celecoxib and other *Cox* inhibitors lowers the risk to develop colon, lung, prostate and breast cancer [252, 253]. However, cardiovascular problems after regular uptake of some selective *Cox-2* inhibitors and general gastrointestinal side-effects associated with NSAIDs impede recommendations of *Cox* inhibitors for colon cancer prevention in the general public [251, 253].

Difluoromethylornithine (DFMO) is an irreversible inhibitor of *ornithine decarboxylase* (*ODC*), the first and rate-limiting enzyme in polyamine synthesis. Polyamines are essential for cell proliferation, but *ODC* is often overexpressed in tumor tissue [254]. DFMO has been shown to inhibit carcinogen-induced tumor formation in a number of rodent models, and has been tested in clinical trials for prevention of colon, skin, cervical and bladder cancer [255]. Meyskens *et al*. recently demonstrated that the combination of DFMO and the NSAID sulindac was remarkably effective in preventing recurrence of sporadic adenomatous polyps, underlining the promise of combination chemoprevention [256].

Pereira *et al.* addressed the question whether celecoxib and DFMO would affect DNA methylation in a rodent

model of azoxymethane-induced colon tumors. The compounds were applied for 1 or 4 weeks prior to termination of the study, when tumors had already established. Intervention with both compounds alone and in combination reversed global DNA hypomethylation, reduced DNA hypermethylation in the *ERα* promoter region and exon 1, and led to *ERα* mRNA reexpression. The mechanism underlying this dual effect on aberrant DNA methylation during carcinogenesis was not further investigated [257]. Similar reversal of DNA hypomethylation in colonic tumor tissue was observed after intervention with $CaCl_2$ and the NSAID piroxicam for 1 week, but not with a series of other compounds lacking colon cancer preventive efficacy in this model [258].

4.7.2. 5-Aza-2'-Deoxycytidine and Zebularine

5-Aza-2'-deoxycytidine is a nucleoside *DNMT* inhibitor used in the clinics to treat hematologic malignancies [259]. 5-Aza-2'-deoxycytidine and other nucleoside analogs are incorporated into DNA during replication. *DNMTs* can not discriminate between cytosine and 5-aza-cytosine residues in the DNA. During the methylation reaction, the 5-aza moiety prevents cleavage of a covalent reaction intermediate, thereby irreversibly trapping *DNMT* as a DNA-adduct. Consequently, *DNMT* levels are depleted, and DNA becomes hypomethylated. DNA demethylating activity of nucleoside *DNMT* inhibitors are associated with substantial toxic effects, limiting their further development as therapeutic agents. The more stable zebularine is structurally related to 5-aza-2'-deoxycytidine and may be less toxic [260].

Both compounds might not been suitable for human cancer prevention due to their apparent toxicity. However, as described above, they were effective in inhibiting tumor formation in several rodent models (summarized in Supplementary Table **1**). If one can assume that the effects on tumor growth are associated with modulation of DNA methylation, results from these studies provide relevant information for the design of intervention studies with natural chemopreventive agents targeting the epigenome.

4.8. Chemopreventive Compounds Affecting SIRT Activity

4.8.1. The Complex Role of SIRT1 in Carcinogenesis

Human class III *HDAC*s, also known as sirtuins, include seven proteins named *SIRT1–SIRT7*. In contrast to class I and II *HDACs*, sirtuin-mediated deacetylation activity is NAD^+-dependent. *Silent information regulator 1* (*SIRT1)* is involved in a variety of cellular processes, from fat and glucose metabolism to ageing and cancer (recent review in [261]). Its role in carcinogenesis is still poorly understood (recent perspective in [262]). *SIRT1* expression is tightly controlled by several negative feedback loops: It is under the control of transcription factor *E2F*, known to induce the transcription of pro-apoptotic genes such as *p53* after DNA damage. However, *E2F* is also a substrate of *SIRT1*, and de-acetylation inhibits its activity as a transcription factor. *p53* can directly repress *SIRT1* transcription; on the other hand, *p53* de-acetylation through *SIRT1* prevents *p53*-mediated transactivation of cell cycle inhibitor *p21* and pro-apoptotic *Bax*, allowing promotion of cell survival after DNA damage and ultimately tumorigenesis. The transcriptional repressor and tumor suppressor gene *HIC1*, which is often hypermethylated in cancer, also controls *SIRT1* expression. Loss of *HIC1* through promoter methylation resulted in overexpression of *SIRT1* in murine breast and prostate cancer cells (cited in [262]). *SIRT1* has a repressive effect on genes involved in stress response, including *p53* and members of the *FOXO* transcription factor family. De-acetylation of *FOXO3* in response to DNA damage provides resistance to oxidative stress, promotes the induction of cell cycle arrest and reduces *FOXO*-mediated apoptosis to allow DNA repair. Continuous bypass of apoptosis can however contribute to tumorigenesis in cells that accumulate DNA damage. On the other hand, *SIRT1*-deficient cells demonstrated an impaired DNA damage response, suggesting that *SIRT1* helps cells to recover from DNA damage. This is even mediated by relocalization of *SIRT1* from repetitive sequences to sites of DNA breaks in response to DNA repair (cited in [262]). Accumulating evidence suggests that the effects of *SIRT1* depend on the genetics of a specific tumor and the presence or absence of *p53*. *SIRT1* overexpression seems to be oncogenic in tumors expressing wt *p53*, but has tumor-suppressive functions in tumors with mutated *p53*. Still, *SIRT1* does not display classical characteristics of a tumor suppressor gene. Neither was it described to accumulate mutations or is inactivated by gene deletions, nor does it induced cell growth arrest when expressed in cell culture. However, in the $APC^{Min/+}$ mouse model, increased *SIRT1* levels resulted in reduced cell proliferation and reduced tumor formation. A study by Holloway *et al*. has recently established that *SIRT1* is an important regulator of *Wnt* signaling, and that inhibition of *SIRT1* leads to changes in expression of *Wnt* target genes. The fact that *Wnt* signaling regulates diverse processes might explain the pleiotropic effects of *SIRT1* [263].

4.8.2. Resveratrol – a SIRT1 Activator

Interest in identifying activators of *SIRT1* was raised when the chemopreventive agent resveratrol from grapes and red wine was described to activate the function of *SIRT1* and to increase lifespan in yeast and *C. elegans* [264], mimicking the effects of calorie restriction [265].

Chemopreventive activity of resveratrol was first described in 1997 [266]. Since then, resveratrol has become one of the most intensely investigated chemopreventive agents (extensive review in [267]). It exhibits pleiotropic health beneficial effects including anti-oxidant, anti-inflammatory, cardioprotective and anti-tumor activities. Resveratrol inhibits experimental tumorigenesis in models for breast, skin, esophageal and colon cancer, and reduced tumor growth in series of xenograft experiments with various tumor cell lines. Resveratrol has been described to target hormone receptors, modulate drug metabolism, and affect many components of intracellular signaling pathways including pro-inflammatory mediators, regulators of cell survival and apoptosis, tumor angiogenesis and metastasis [267, 268]. Despite reported *in vivo* efficacy, plasma levels after oral consumption are extremely low [269]. Resveratrol is currently undergoing clinical testing in several small intervention trials [267].

A recent report adds a new aspect to the multiple mechanisms elicited by resveratrol. Tili *et al.* investigated

potential of resveratrol to modulate miRNA expression in colon cancer cells. Analyses with miRNA microarrays indicated that resveratrol-treatment for 14 h significantly upregulated the expression of 22 miRNAs, while 26 miRNA were downregulated. Several downregulated miRNAs, such as *miR-17*, *miR-21*, *miR-25*, *miR-92a-2* were known to be upregulated in colon cancer. Resveratrol increased levels of *miR-663*, a microRNA with tumor-suppressor functions that targets *TGFβ1* transcripts. Resveratrol treatment upregulated several components of the *TGFβ1* signaling pathway, including *TGFβ1* receptors type I and type II, whereas transcriptional activity of *SMAD* proteins as key effectors of the canonical *TGFβ* pathway were downregulated. These findings suggest that manipulation of the levels of key microRNAs, such as m*iR-663,* may contribute to the anti-cancer and anti-metastatic effects of resveratrol [270]. In a second study, the effect of resveratrol on miRNA expression was analyzed in THP-1 monocytic cells, to investigate a potential role in adaptive and innate immune response. In THP-1 cells, *miR-663* decreased endogenous activator protein 1 (*AP-1)* activity in part by directly targeting *JunB* and *JunD* transcripts, and impaired its upregulation by LPS. Similar to the effects in colon cancer cells, resveratrol treatment of THP-1 cells upregulated *miR-663* expression. Resveratrol also downregulated *AP-1* activity, and this was shown to be *miR-663* dependent. Further, resveratrol impaired the LPS-mediated upregulation of *miR-155* in THP-1 cells in a *miR-663*-dependent manner [271].

4.8.3. Dihydrocoumarin and Cambinol – SIRT Inhibitors

In line with the reported anti-apoptotic effect of *SIRT1* under stress conditions through deacetylation of key factors such as *p53* and *FOXO3a*, reversal of these effects by *SIRT* inhibition should induce apoptosis. Dihydrocoumarin and cambinol have been identified as *SIRT* inhibitors, but they have not been tested for chemopreventive potential yet.

Dihydrocoumarin is a dietary compound found in *Melilotus officinalis* (sweet clover) and is frequently used as a flavoring agent in food supplements and in cosmetics. Olaharski *et al*. first described that DHC inhibited the deacetylase activities of yeast *Sir2p* and human *SIRT1*. Exposure of human TK6 lymphoblastoid cells to DHC led to dose-dependent increases of *ac-p53*, cytotoxicity, and apoptosis [272].

Cambinol is a β-naphthol compound that was identified as an inhibitor of *SIRT1* and *SIRT2* in a chemical screen [273]. Kinetic studies indicated that cambinol was competitive with the histone H4 peptide and non-competitive with NAD^+, and it did not inhibit class I and II *HDAC*s. To demonstrate functional activity in cell culture, NCI H460 lung cancer cells were treated with cambinol. Hyperacetylation of *p53* as a SIRT target protein was seen after inducing DNA damage by co-treatment with etoposide, and etoposide-induced *p21* up-regulation (as a *p53* target) was further promoted by cambinol. Other factors acetylated after cambinol-treatment included *FOXO3a* and *Ku70*. Deacetylation of these proteins promotes cell survival under stress, indicating that inhibition of SIRT with cambinol abrogates several sirtuin-dependent cellular survival pathways. Transcriptional repressor *BCL6* is another sirtuin target. Treatment of *BCL6*-expressing Burkitt lymphoma cells with cambinol induced apoptosis, accompanied by hyperacetylation of *BCL6* and *p53*. *In vivo* analysis of cambinol exposure at 100 mg/kg i.v. or i.p. was well tolerated in mice and inhibited growth of Burkitt lymphoma xenografts in SCID mice [273].

4.9. Modulators of HAT Activity

Several natural products have been identified as inhibitors of histone acetyltransferases (*HAT*), including anacardic acid, garcinol [274], the hydrophilic bile acid ursodeoxycholic acid, and curcumin (see above). On the other hand, genistein has been reported to induce expression of several *HATs* (see above) [160]. The functional consequence of *HAT* inhibition or induction might strongly depend on the cellular context and additional interacting factors.

4.9.1. Anacardic Acid

Anacardic acid (6-nonadecyl salicylic acid) has been isolated from cashew nut shell liquid. Anacardic acid was identified as the first natural product inhibitor of *p300 HAT* activity based on a screen of plant extracts known to possess anticancer properties. Using purified proteins as an enzyme source, it was found to inhibit *p300* and *PCAF* activities with IC_{50} values of 8.5 μM and 5 μM, respectively [275]. *Tip60* plays an important role in activating *ATM* and *DNA-PKcs* as key enzymes in DNA damage response. Anacardic acid inhibited *Tip60 HAT* activity *in vitro* with an IC_{50} value of 9 μM. It also blocked the activation of *ATM* and *DNA-PKc* in cell culture and thereby sensitized tumor cell lines to ionizing radiation [276]. Anacardic acid also interfered with *NF-κB* signaling, which is involved in radiosensitization, as well as in inflammation and tumorigenesis [277]. In a study by Sung *et al*., treatment of various human cancer cell lines with anacardic acid potentiated *TNF-α*-, cisplatin- and doxorubicin-mediated apoptosis induction, and activation of caspases by *TNF-α*. Anacardic acid strongly suppressed the *TNF-α*-mediated upregulation of anti-apoptotic proteins, including *Bcl-2, Bcl-xL, cFLIP, cIAP-1*, and *survivin*, as well as of *cyclin D1, c-Myc, Cox-2, VEGF, ICAM*-1, and *MMP9* involved in invasion and angiogenesis. All of these factors are regulated by *NF-κB*. Inhibition of *NF-κB* activation by *TNF-α* and a series of other stimuli was demonstrated by EMSA experiments. As a mechanisms, anacardic acid was found to repress the activation of *IκBα*, and suppressed acetylation and nuclear translocation of *NF-κB* subunit *p65*. All of these effects could be mimicked by down-regulation of *p300 HAT* by siRNA, indicating that *p300* is a key mediator of the effects of anacardic acid on *NF-κB* signaling. These findings suggest that anacardic acid might be an interesting lead compound for further development in cancer prevention [277].

4.9.2. Garcinol

Garcinol is a polyisoprenylated benzophenone isolated from the Mangosteen tree *Garcinia indica* Choisy (*Clusiaceae*). Garcinol was shown to act as a potential chemopreventive agent by anti-oxidant activity, induction of phase II detoxifying enzymes, anti-inflammatory effects, inhibition of cell proliferation, and induction of apoptosis. *In vivo*, it prevented AOM-induced aberrant crypt foci in rat colon, and 4-NQO-induced tongue carcinogenesis (review in [278]). Garcinol was identified as a cell-permeable *HAT*

inhibitor which inhibits *PCAF* and *p300 HAT* activity with IC_{50} values of 5 μM and 7 μM, respectively. Garcinol repressed general histone acetylation in HeLa cells and induced apoptosis [279]. Garcinol potentiated *TRAIL*-induced apoptosis of cancer cells by up-regulation of the *TRAIL* receptors death receptor 4 (*DR4)* and *DR5*. This effect was abrogated by deletion of *DR5* or *DR4* by siRNA. Similar to the activities of anacardic acid, garcinol reduced the expression of various anti-apoptotic proteins, including *survivin*, *Bcl-2*, *XIAP*, and *cFLIP*. Apparently, the activities of garcinol are mediated through the generation of reactive oxygen species, since all of the effects were abolished by pre-treatment with N-acetyl cysteine [280]. Garcinol has previously been reported to inhibit *NF-κB*, but potential inactivation of *NF-κB* through reduced acetylation of *p65* was not analyzed in this study.

4.9.3. Ursodeoxycholic Acid (UDCA)

Ursodeoxycholic acid (UDCA, ursodiol) is an endogenous hydrophilic bile acid. In chemically-induced rodent models for colon cancer, UDCA prevented tumor development by abrogating the tumor-promoting effects of secondary bile acids such as deoxycholic acid. Retrospective and prospective epidemiological studies have suggested colon cancer preventive potential (reviewed in [281]). Earlier studies have defined a cell-protective mechanism of UDCA by inhibition of classical pathways of apoptosis induction (reviewed in [282]). Recent mechanistic studies indicate that UDCA reduced histone acetylation and induced cell differentiation and senescence in colon cancer cells. Differentiation was indicated by altered morphology, *cytokeratin* and *E-cadherin* upregulation, cytoskeleton rearrangement, and accumulation of lipid droplets. In contrast to butyrate, UDCA did not induce intestinal alkaline phosphatase as a differentiation marker. Also, it did not induced histone hyperacetylation, but reduced global histone acetylation, indicating that both compounds induce differentiation by distinct mechanisms. Subsequent inhibition tests confirmed that UDCA is not an *HDAC* inhibitor, nor did it directly inhibit *HAT* activity. Instead, it induced *HDAC6* expression. Signs of senescence in UDCA treated cells included a flat, vacuolated morphology and β-galactosidase staining. Interestingly, *HDAC6* overexpression was sufficient to induce senescence and might play an important role in UDCA-mediated effects. These data indicate that butyrate and UDCA influence chromatin acetylation in different directions. UDCA should not be combined with butyrate or other *HDAC* inhibitors for colon cancer prevention [283].

4.10. Modulators of Histone Lysine Methylation

The chromatin is dynamically regulated by histone acetylation, methylation and other posttranslational modifications. *HDAC* inhibitors have been intensely investigated and show promise for the treatment and prevention of cancer. So far, only a few chemopreventive agents with potential to affect histone lysine methylation have been described, including n-3 polyunsaturated fatty acids (n-3 PUFA) and EGCG, which was shown to downregulate *BMI-1, SUZ12*, and *EZH2* expression in various cancer cell lines, leading to reduced H3K27 trimethylation, cell cycle arrest and apoptosis induction (described above) [147]. In addition, the alkaloid chaetocin and polyamine analogues (PG11144) have been shown to modulate histone lysine methylation and might represent interesting candidates for chemoprevention.

4.10.1. Chaetocin

Chaetocin is a fungal metabolite, first isolated in 1970, with a complex epidithiodiketopiperazine alkaloid structure. Chaetocin is one of the first identified selective inhibitors for the *SUV39* class of histone lysine methyltransferases targeting H3K9 (reviewed [284]). H3K9 trimethylation is generally associated with repressed chromatin. Chaetocin has antibacterial and cytostatic activity, but has not been investigated with respect to cancer prevention yet. Isham *et al.* demonstrated that chaetocin has anti-myeloma activity mediated by generation of oxidative stress and apoptosis induction, without affecting normal B-cells and chronic lymphocytic leukemia cells [285]. Recently, Cherrier *et al*. established that *CTIP2* (*COUP-TF-interacting protein 2*) is recruited to the promoter of cell cycle inhibitor *p21* and transcriptionally silences *p21* expression through interactions with *HDACs* and histone methyltransferases. Chaetocin treatment of microglial cells transfected with a *p21*-promoter reporter construct repressed H3K9 trimethylation at the *p21* promoter, stimulated *p21* expression, and induced cell cycle arrest [286]. Lakshmikuttyamma *et al*. established a link between *SUV39* inhibition and re-expression of $p15^{INK4B}$ and *E-cadherin* epigenetically silenced in acute myeloid leukemia (AML). Treatment of various AML cell lines with the DNMT inhibitor 5-aza-2'-deoxycytidine induced $p15^{INK4B}$ and *E-cadherin* expression with concomitant reduction of promoter methylation, H3K9 methylation and *SUV39* associated with the promoter regions. In contrast, incubation with chaetocin induced H3K9 demethylation and activated $p15^{INK4B}$ and *E-cadherin* re-expression without promoter demethylation. These activities were mimicked by down-regulation of *SUV39* with shRNA. Inhibition of *SUV39* by chaetocin treatment or shRNA reduced cell proliferation and led to the induction of apoptosis. These data indicate that inhibitors of *SUV39* and H3K9 methylation may be interesting candidates for reactivating expression of hypermethylated genes [287].

4.10.2. Polyamine Analogues-PG11144 and PG11150

Histone lysine methylation is a dynamic process that is maintained and regulated by the interplay of histone methyltransferases on one hand, and demethylases on the other hand. *Lysine-specific demethylase 1* (*LSD1*) is a FAD-dependent amine oxidase, which was shown to interact directly with *HDAC1/2* proteins in multiprotein co-repressor complexes. *LSD1* demethylates mono- and di-methylated H3K4 through an oxidative reaction that leads to the formation of H_2O_2 and formaldehyde. Through demethylation of H3K4me2/me1 associated with active chromatin, *LSD1* has the potential to broadly repress gene expression ([288] and references cited therein). High homology of *LSD1* with mono- and polyamine oxidases, and structural similarities of polyamines with histone lysine residues led to the hypothesis that polyamines might inhibit *LSD1* activity. Treatment of colon cancer cells with polyamine analogues indeed resulted in re-expression of silenced tumor suppressor genes, including members of the *secreted frizzle-related proteins* (*SFRP*s) and the *GATA* family of transcription factors [289]. *SFRP*s are negative regulators of the *Wnt-*

signaling pathway. In a recent screen to identify additional *LSD1* inhibitors, two decamine analogues, PG11144 and PG11150 were identified as potent competitive inhibitors with IC_{50} values of 5 μM. Both compounds inhibited colon cancer cell proliferation and induced apoptosis. Global H3K4 mono- and di-methylation was induced in a concentration dependent manner, concomitant with *SFRP1* and *SFRP2* mRNA expression. Intervention also led to changes in H3K9 methylation and H3K9 and H4K16 acetylation. The effects on *SRFP2* re-expression were enhanced by co-incubation with 5-aza-2'-deoxycytidine. Combined treatment with PG-11144 and 5-aza-2'-deoxycytidine strongly repressed tumor growth of HCT116 colon cancer xenografts [288]. Based on these findings, *LSD1* inhibitors represent an interesting class of compounds for future preventive and therapeutic approaches.

4.10.3. n-3 Polyunsaturated Fatty Acid (n-3 PUFAs)

n-3 (also known as omega-3) polyunsaturated fatty acids (PUFAs) are essential for human health and have to be consumed with the diet. α-Linolenic acid is a plant-derived n-3 PUFA found for example in soybeans, walnuts, dark green leafy vegetables and seed oils. In general, cold-water fish (fish-oil) are the main source for dietary long-chain n-3 PUFAs, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Epidemiological studies suggest a protective effect of n-3 PUFAs on colon and prostate cancer development, but the evidence is somewhat mixed. In contrast, so called n-6 PUFAs, such as linoleic acid or arachidonic acid, have been associated with tumor promotion. In rodent models for cancer prevention, n-3 PUFAs have been consistently shown to reduce prostate, breast and colon cancer. Mechanisms involved in protection include anti-inflammatory activity, effects on signaling pathways, and inhibition of tumor growth, invasion, and angiogenesis. Several ongoing clinical trials currently investigate potential of n-3 PUFAs to prevent cancer development of colon, prostate, breast and several other cancer types, or to support and treat cancer patients (summary in [290]).

Recent studies now provide a link between n-3 PUFAs and epigenetic mechanisms. Dimri *et al.* investigated the potential of n-3 PUFAs to modulate histone modifications in breast cancer, and identified *EZH2* as a target of regulation by n-3 PUFAs [291]. *EZH2* maintains the transcriptional repressive state of genes by promoting H3K27 trimethylation. It is overexpressed in various cancer types including breast and prostate cancer. Human breast cancer cells were treated with individual n-3- and n-6 PUFAs. Only n-3-, but not n-6 PUFAs were able to reduce the expression of *EZH2*. Repression was mediated at the post-translational level by increasing proteasomal degradation of *EZH2*, and was associated with reduced levels of H3K27me3. Expression of *E-cadherin* and *IGFBP3* (*insulin-like growth factor binding protein 3*), which are known targets to *EZH2*, were up-regulated by n-3 PUFA treatment, accompanied by a decreased invasive phenotype. These studies establish *EZH2* as an interesting target for chemoprevention [291].

Recent data by Davidson *et al*. indicate that intervention with n-3 PUFA has an impact on carcinogen-induced dysregulation of miRNAs in rat colon [292]. Rats were fed diets containing n-3- (fish oil) or n-6 PUFAs (corn oil) in combination with pectin or cellulose. Tumors were induced by two weekly injections with azoxymethane. Of the 153 miRNAs detected in rat colon, 27 were upregulated in tumor tissue, and 19 were downregulated in comparison with normal mucosa. Each diet combination had a distinct effect on miRNA expression, as indicated by cluster analyses. Downregulation of five miRNA (*let-7d*, *mir-15b*, *miR-107*, *miR-109* and *miR-324-5p)* by AOM-treatment was selectively prevented by fish oil exposure. Overall, fish oil fed animals showed the lowest number of differentially expressed miRNAs. Also, fish-oil intervention was most effective in reducing numbers of AOM-induced tumors. In transfection experiments with HCT116 colon cancer cells, tumor suppressor *PTEN* and *BACE1* (*beta-site amyloid precursor protein-cleaving enzyme 1)* were confirmed as functional targets of *miR-21* and *miR-107*, that were up- and down-regulated, respectively, in tumor tissue versus normal colonic mucosa. This study demonstrates that dietary n-3 PUFA can protect from carcinogen-induced changes in miRNA profiles [292]. Overall, these data add to our understanding of the chemopreventive potential of n-3 PUFAs.

5. SUMMARY AND OUTLOOK

As outlined above, chemopreventive agents target the epigenome by multiple and interacting pathways. Their effects on global DNA methylation, tumor suppressor genes silenced by promoter methylation, histone modifications, and miRNAs deregulated during carcinogenesis have potential impact on mechanisms relevant for chemoprevention, including signal transduction mediated by nuclear receptors and transcription factors such as *NF-κB*, cell cycle progression, cellular differentiation, apoptosis induction, senescence and others. A simplified overview of most prominent epigenetic mechanisms elicited by chemopreventive agents, and key factors influenced by modulation of these mechanisms is given in Fig. (**3**).

Most of the chemopreventive agents described in this review possess chemopreventive potential in various genetic- or carcinogen-induced tumorigenesis models, and there is ample and convincing evidence that these agents have potential to regulate gene expression by epigenetic mechanisms, at least in cell culture systems. Future investigations in animal models will have to demonstrate that these observations are functionally linked, and will clarify whether chemopreventive efficacy is mediated by epigenetic gene regulation, or is based on other chemopreventive mechanisms, or likely a combination of both. Some of the described effects on epigenetic gene regulation appear to be cell type or organ-specific, but in most cases, underlying mechanisms for these differences have not been addressed yet. Also, most investigations on epigenetic effects so far have only been performed in a targeted candidate gene approach. Future investigations on the modulation of DNA methylation and histone modifications at a genome-wide level will help to better understand mechanistic links.

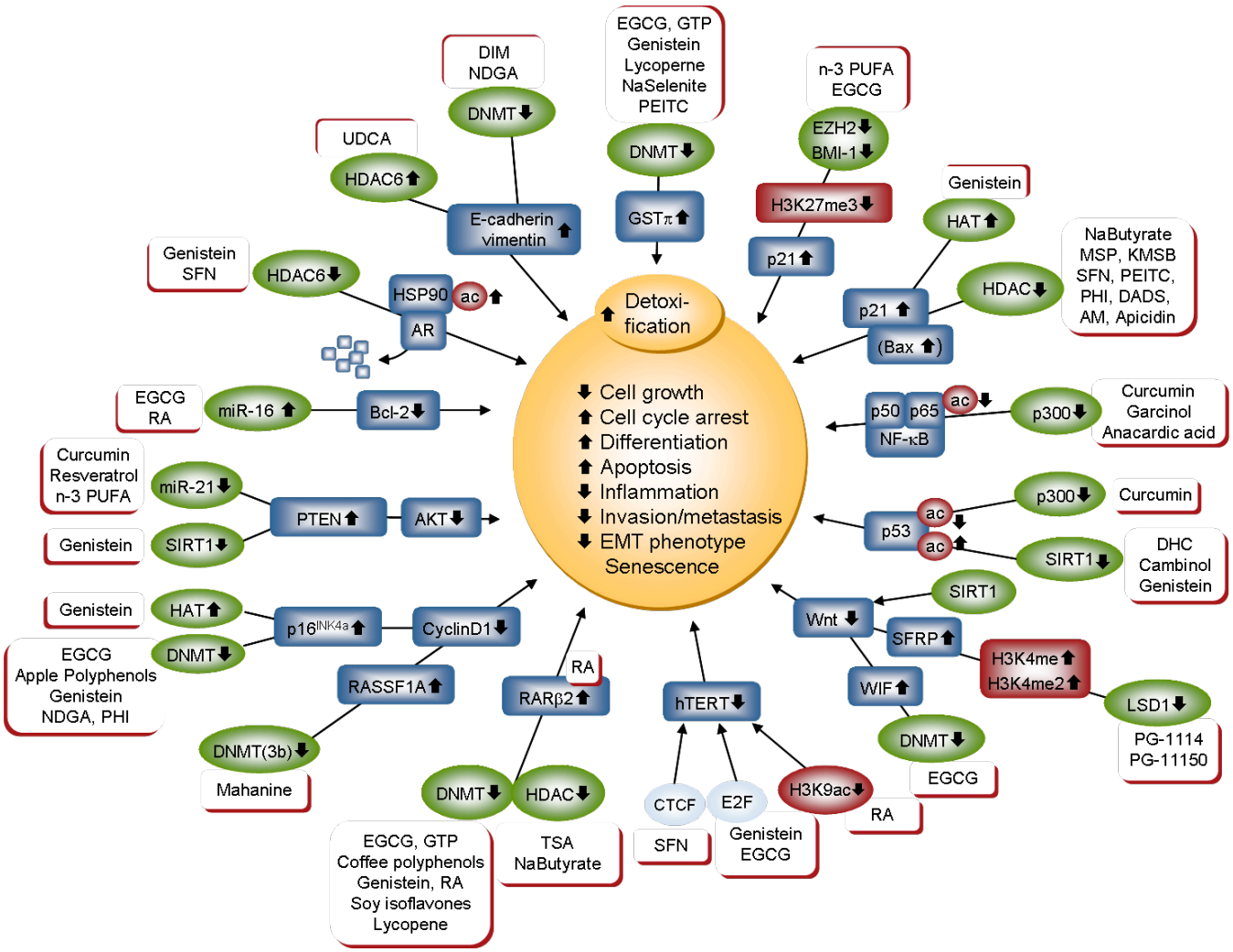

Fig. (3). Overview of epigenetic mechanisms targeted by chemopreventive agents, and their influence on pathways and mechanisms controlling tumor growth.

Abbreviations: AM, allyl-mercaptan; BMI-1, B-cell-specific Moloney murine leukemia virus integration site 1 (histone lysine methyltransferase); DADS, diallyldisulfide; DHC, dihydrocoumarin; DIM, diindolylmethane; EGCG, epigallocatechin gallate; EZH2, enhancer of zeste 2 (histone lysine methyltransferase); GTP, green tea polyphenols; HAT, histone acetyl transferase (e.g. p300); KMSB, α-keto-γ-methylselenobutyrate; MSP, β-methylselenopyruvate; NDGA, nordihydroguaiaretic acid; PEITC, phenethyl isothiocyanate; PHI, phenhexyl isothiocyanate; n-3 PUFA, n-3 polyunsaturated fatty acids; RA, retinoic acid; SITR1, Silent information regulator 1 (NAD^+-dependent histone deacetylase class III); SFN, sulforaphane; SFRP, secreted frizzled-related protein; TSA, trichostatin A; UDCA, ursodeoxycholic acid ; WIF, Wnt-inhibitory factor.

Another point to consider in future chemoprevention studies is that timing of intervention might be critical to target epigenetic deregulation during tumorigenesis. Global DNA hypomethylation and promoter hypermethylation have been identified as early events during cancer development. If intervention with chemopreventive agents is started only after critical events have taken place, a modulating effect would likely be missed. As mentioned above, 5-aza-2'-deoxycytidine prevented intestinal neoplasia in *APC*$^{Min/+}$ mice by >95% when intervention was started at the age of 1 week of age, whereas the compound was ineffective when application started at 7 weeks of age [65]. In studies with chemopreventive agents, intervention often has not be started much before this age. Therefore the contribution of epigenetic mechanisms to chemopreventive efficacy in rodent models might have been underestimated so far. The question of 'critical time windows' for application should be addressed in more detail in the future, both in direction of cancer prevention, but also with respect to potential harmful effects. Along these lines, frequency of application might also be a critical determinant of chemopreventive efficacy. Inhibition of *HDACs* by dietary agents such as ITCs and butyrate and consequent histone hyperacetylation is a transient effect. Although these activities have been demonstrated in rodent models and in humans, so far it is not clear whether occasional consumption of dietary *HDAC* inhibitors in form of cruciferous vegetables or dietary fiber as a precursor of butyrate would result in long-term epigenetic regulation of gene expression and downstream chemopreventive effects. This also applies to other epigenetic mechanisms.

As outlined above, some interventions are more effective when applied in combination. The concept of combining *DNMT* and *HDAC* inhibitors to regulate gene expression in cancer therapy is not new, as demonstrated by the combined application of RA with *DNMT* or *HDAC* inhibitors. This aspect has not been systematically investigated with dietary agents affecting the epigenome (some examples in [133]), but might be relevant when comparing activities of isolated compounds with complex extracts or food items. As mentioned above, some combinations even appear to be contraindicated, such as UDCA together with butyrate or other *HDAC* inhibitors.

It becomes more and more clear that epigenetic gene regulation involves a crosstalk between effects on DNA methylation, histone modifications and miRNAs expression, coordinated in an intricate network. Most studies on chemopreventive agents targeting the epigenome so far have focused on one particular epigenetic mechanism, for example re-expression of tumor suppressor genes silenced by DNA hypermethylation. To fully understand the potential impact of epigenetic gene regulation and to target it for chemoprevention, we need to consider the epigenome as an interactive three dimensional system. With the development of methodologies for genome-wide assessment of DNA methylation and localization of activating or repressing histone marks, high throughput assays for quantitative determination of DNA methylation, more validated targets of miRNAs, and bioinformatic tools to systematically integrate available information, systems are available to address these open questions in future chemoprevention studies.

SUPPLEMRNTRY MATERIAL

This article is also accompanied with supplementary material and it can be viewed online at publisher's web site.

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