Epigenetic impact of dietary isothiocyanates in cancer chemoprevention

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Purpose of review
There is growing evidence that cancer chemopreventive agents including isothiocyanates (ITCs) from cruciferous vegetables target epigenetic mechanisms. The present report will summarize novel findings of ITCs on histone deacetylase activity, DNA methylation, and short noncoding microRNAs, focusing on sulforaphane (SFN) from broccoli and phenethylisothiocyanate from watercress.

Recent findings
In a human intervention study, broccoli sprouts led to more efficient histone deacetylase inhibition in blood cells than a broccoli sprout supplement, correlating with higher levels of urinary ITC metabolites. A proteomics study with 14C-labeled ITCs revealed among others a direct interaction with histones and chromatin-modulating proteins. The well investigated Kelch-like erythroid-cell-derived protein with CNC homology-associated protein 1/nuclear factor erythroid 2-related factor 2/antioxidant-response element pathway is both affected by and mechanistically involved in epigenetic activities of ITCs. Accordingly, reduction of oxidative stress is shown to prevent hypertension-associated global hypomethylation in rats. Combination of SFN with (−)-epigallocatechin gallate as a demethylating agent is identified as an effective approach for re-expression of estrogen receptor in hormone negative breast cancer. Induction of miR-200c by SFN prevents epithelial–mesenchymal-transition and could be relevant for prevention of metastases.

Summary
The last year has identified interesting areas of ITCs affecting epigenetic mechanisms that will have implications for translational cancer (prevention) research once validated in animal studies and human intervention studies.

Keywords
DNA methylation, epithelial–mesenchymal-transition (EMT), histone deacetylase (HDAC), KEAP1/Nrf2/ARE pathway, microRNA, phenethylisothiocyanate (PEITC), sulforaphane (SFN)

INTRODUCTION
Epigenetics describes heritable, but potentially reversible, alternations in gene expression without modifications in the DNA sequence. Epigenetic mechanisms comprise post-translational histone modifications (including, but not limited to, histone acetylation and methylation), DNA hypomethylation and hypermethylation, and post-transcriptional regulation of gene expression by noncoding microRNAs (miRNAs). As epigenetic aberrations occur early in carcinogenesis and represent potentially initiating events in cancer development, they can be regarded as promising new targets for cancer prevention strategies (overview in [1*]). Research over the last 10 years has revealed that many cancer chemopreventive agents influence activity and expression of DNA methyltransferases (DNMTs) mediating DNA methylation, histone acetyltransferases and histone deacetylases (HDACs) controlling chromatin accessibility through acetylation of histone tails, and miRNAs, each of which might target expression of hundreds of target genes. It has been postulated that these effects on epigenetic mechanisms might be intricately associated with their broad spectrum of chemopreventive actions [1*,2].

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Isothiocyanates (ITCs) from cruciferous vegetables, such as sulforaphane (SFN) from broccoli (sprouts) and phenethylisothiocyanate (PEITC) from watercress were discovered as chemopreventive agents more than 30 years ago (overview in [3]). In the plant, they are stored at high concentrations in the form of glucosinate precursors, sugar bound compounds that give rise to ITCs upon chewing, cutting, or microbial damage, catalyzed by the enzyme myrosinase. Chemopreventive efficacy of ITCs has been shown in various rodent models of carcinogen-induced or transgene-induced carcinogenesis. Best investigated mechanisms of action include modulation of cytoprotective bio-transformation enzymes via the Kelch-like erythroid-cell-derived protein with CNC homology (ECH)-associated protein 1 (KEAP1)/Nuclear factor erythroid 2-related factor 2 (Nrf2)/Antioxidant response element (ARE) pathway, anti-inflamatory activity via inhibition of nuclear factor kappa B (NF-kB), inhibition of proliferation through induction of cell cycle arrest and programmed cell death (apoptosis), modulation of hormone receptor expression, antiangiogenic and antimetastasis potential, and induction of autophagy (overview in [3,4]). Several clinical trials with SFN, PEITC, watercress juice, or broccoli sprouts have been initialized for prevention or treatment of lung, oral, breast, or prostate cancer, or lymphoproliferative disorders (http://www.cancer.gov/clinicaltrials).

**ISOTHIOCYANATE METABOLITES INHIBIT HISTONE DEACETYLASE ACTIVITY**

Potential of ITCs to target the epigenome was first discovered in 2004 by the group of Rod Dashwood from the Linus Pauling Institute in Oregon. They found that a metabolite of SFN, the major ITC derived from broccoli, inhibited HDAC activity and led to histone hyperacetylation in cell culture and in vivo (overview in [5]). In a recent study, the same group performed a small human pilot study with 11 men and 11 women participants to compare bioavailability and HDAC inhibitory potential of broccoli ITCs provided in form of fresh broccoli sprouts (68 g/day), or as a commercially available broccoli sprout supplement containing a total of 3 g freeze dried broccoli sprouts [6]. Although total amounts of glucosinolates were similar in both preparations, the extent and kinetics of urinary excretion of ITC metabolites largely differed between both intervention groups, with faster excretion and five-fold to eight-fold higher levels of major ITC metabolites in the sprout group. These differences were attributed to the presence of active myrosinase in the sprout preparation. Consistent with lower levels of metabolite excretion, HDAC activity in white blood cells was not inhibited in the supplement group [6].

**DIRECT BINDING OF ISOTHIOCYANATES TO CHROMATIN-ASSOCIATED PROTEINS**

ITC chemically belong to the class of thiol-reactive Michael acceptors. Part of their reactivity has been associated with their potential to interact with and covalently bind to protein thiol groups via thio carbamoylation. To identify proteins intracellularly targeted by ITCs, Mi et al. [7] treated human lung cancer cells with radioactivity-labelled 14C-SFN and 14C-PEITC [7]. Using 2D-gel electrophoresis and mass spectrometry in a proteomic approach, they identified more than 30 proteins bound by the ITCs. Beside cytoskeletal (α-tubulin and β-tubulin, actin) and redox-regulating proteins, proteasome subunits, heat shock, mitochondrial, and signaling regulatory proteins (e.g. 14–3–3 adapter proteins with key roles in cell cycle, apoptosis and regulation of cell survival), and heterogeneous nuclear ribonucleoproteins involved in RNA splicing, these include retinoblastoma binding protein 4 important for maintenance of pluripotency in stem cells, SMARCE1, a SWI/SNF-related chromatin remodeler, and histones H2A and H4 as potential ITC targets. This study provides important novel hints for potential direct intracellular targets of ITCs including chromatin modifying proteins, but direct interaction and impact on protein function has to be validated and further elucidated in future studies [7].

**EPIGENETIC MECHANISMS ASSOCIATED WITH THE KEAP1/Nr2/ARE PATHWAY**

The KEAP1/Nrf2/ARE pathway plays an important role in the induction of cytoprotective enzymes and...
has been identified as an important target for chemopreventive agents including ITCs [8]. Chromatin-immunoprecipitation using an antibody against the transcription factor Nrf2 coupled with sequencing of the chromatin-bound DNA (ChIP-Seq) has recently revealed more than 240 genomic regions bound to Nrf2 after stimulation of human lymphoblastoid cells with SFN [9]. The Kong group at Rutgers University had reported that Nrf2 expression was gradually lost during prostate carcinogenesis in the transgenic adenocarcinoma of the mouse prostate (TRAMP) transgenic mouse model for prostate cancer, and identified promoter methylation as the underlying mechanisms for silencing [10]. SFN treatment of TRAMP C1 cells derived from the murine model led to de-repression of Nrf2 by a combined epigenetic mechanism comprising reduced expression of DNA methyltransferase 1 (DNMT1) and DNMT3a, demethylation of specific cytosine-phosphate-guanine (CpG) dinucleotides in the Nrf2 promoter, enhanced histone H3 acetylation (ac-H3) and elevated ac-H3 binding to the Nrf2 promoter, indicating active transcription [10].

In chronic obstructive pulmonary disease (COPD), HDAC2 is deactivated by S-nitrosylation caused by cigarette smoke-mediated activation of NF-κB, leading to induction of proinflammatory proteins including inducible nitric oxide synthase. As a consequence HDAC2 binding and deacetylation of the glucocorticoid receptor are blocked, abolishing the anti-inflammatory effects of corticosteroid treatment [11]. Malhotra et al. reported that SFN-mediated activation of Nrf2 and induction of glutathione led to the reactivation of HDAC2 and re-sensitized macrophages of COPD patients to corticosteroids. The fact that SFN (or its metabolites, respectively) is a HDAC inhibitor was not discussed in this study [11]. SFN and allylisothiocyanate (AITC), derived from Brassica species such as mustard, horseradish and wasabi, reduced lipopolysaccharide-induced NF-κB-mediated transcription of proinflammatory proteins in murine macrophages [12]. Notably, both ITCs dose-dependently reduced the expression of miR-155 that has been strongly linked to the proinflammatory response of macrophages [12]. Whether in addition to glutathione induction SFN and other ITCs might influence COPD and corticosteroid sensitivity by epigenetic mechanisms such as downregulation of miR-155 needs to be addressed in future studies.

Application of broccoli sprouts to spontaneously hypertensive stroke-prone rats (SHRSP) was shown to reduce renal and vascular oxidative stress, inflammation, and blood pressure (cited in [13]). In a follow-up study with SFN, renal pathological abnormalities were reduced and blood pressure improved. Concomitantly, global kidney DNA hypomethylation in SHRSP rats compared with healthy Sprague Dawley rats was normalized by SFN intervention. These data suggest that DNA hypomethylation might be associated with hypertension and could be prevented by reduction of oxidative stress [13], for example, by upregulation of Nrf2 signaling.

As a note of caution, several studies have recently identified even though rare activating mutations in Nrf2 and the chaperone KEAP1 in several human tumor types, indicating that the KEAP1/Nrf2/ARE pathway could also become oncogenic and contribute to chemotherapy resistance [8]. Intervention with Nrf2 inducers in cancer patients should therefore be considered carefully.

**GENE-SPECIFIC INFLUENCE OF SULFORAPHANE ON DNA METHYLATION ALONE AND IN COMBINATION WITH OTHER CHEMOPREVENTIVE AGENTS**

There is limited information on SFN targeting DNA methylation [1]. Hsu et al. [14] investigated demethylating activity of SFN on cyclin D2 involved in cell cycle regulation. SFN treatment of LNCaP prostate cancer cells weakly reduced global DNA methylation as well as methylation of the cyclin D2 promoter containing binding sites for transcription factors c-Myc and Sp1. Concomitantly, transcription of cyclin D2 increased whereas expression of DNMTs 1 and 3b was downregulated.

First proposed more than 30 years ago, the concept of combination chemoprevention by co-treatment with chemopreventive compounds with distinct targets might provide increased efficacy compared to single compound treatment [15]. Barrera et al. [16] investigated DNA demethylating activity of SFN and the structurally related iberin (3-methylsulfinylpropyl-ITC) alone or in combination with selenium compounds in colon cancer cell lines, focusing on estrogen receptor ESR1, cell cycle inhibitor p16, adenomatous polyposis coli gene APC, the repair gene MGMT, and the repetitive element LINE-1. mRNA expression of DNMT1 and 3b was transiently upregulated, and expression of DNMT3a was reduced after longer term intervention. However, these changes in DNMT expression were not associated with changes in DNA methylation by any of the treatments [16].

Epigenetic effects of combinations of SFN with green tea (-)–epigallocatechin gallate (EGCG) were addressed in two subsequent studies by the Tollefsbol group from the University of Alabama
at Birmingham, with the rationale to combine the chromatin-modifying activity of SFN with demethylating properties of EGCG [17**]. Combined treatment was more effective than each single compound alone in re-expressing estrogen receptor in an estrogen receptor-negative breast cancer cell line. This was associated with significant reduction of DNMT expression and activity, as well as of HDAC activity, with strongest effects observed with the combinations, leading to DNA hypomethylation and hyperacetylation at the estrogen receptor promoter region. As a consequence, estrogen receptor-negative breast cancer cells were resensitized to treatment with antiestrogens such as tamoxifen [17**]. If confirmed in animal models for estrogen receptor-negative breast cancer and human trials, these data might have strong implications for the clinical management of hormone receptor-negative breast cancer.

In a study reported by Chen et al. [18*], efficient downregulation of hTERT, the catalytic subunit of human telomerase, and Bcl-2, an antiapoptotic protein, accompanied by induction of cell cycle arrest and apoptosis, was induced in paclitaxel-resistant ovarian cancer cells by a combination of SFN and EGCG [18*]. DNMT1 expression in the ovarian cancer cell lines was reduced with most pronounced effects by the combination of both compounds. The Tollefsbol group has shown previously that hTERT expression is regulated by DNA methylation at specific promoter CpG sites. Hypomethylation at these sites allows interaction with repressive complexes, leading to hTERT downregulation (summary in [1*]). Chen et al. [18*] did however not determine DNA methylation levels at the hTERT promoter in this study.

Overall, evidence that SFN is affecting gene regulation via DNA demethylation is still weak. The described effects could indirectly be influenced by changes in histone acetylation, chromatin accessibility, or composition of DNA binding complexes. These possibilities need to be systematically addressed.

**MicroRNAs as Targets of Sulfuraphane and Phenethyl Isothiocyanate**

Development of metastases is the major cause of cancer related deaths. During epithelial-to-mesenchymal transition (EMT), epithelial cells undergo a series of biochemical changes to become migratory and invasive, leading to metastases formation [19]. MicroRNAs have been shown to be intricately involved in this process [20*]. In a recent study, Shan et al. [21**] investigated the potential of SFN to reduce EMT in bladder cancer cells, with the rationale that ITC metabolites accumulate to high concentrations in the bladder. SFN-mediated inhibition of migration and invasion was associated with downregulation of cyclooxygenase 2 (Cox-2) and matrix metalloproteinases 2 and 9. Expression of E-cadherin as a marker of epithelial cells was upregulated, whereas expression of mesenchymal markers (ZEB-1, Snail) was reduced. This was associated with a strong increase in miR-200c levels, and cotreatment with a miR-200c inhibitor abrogated the effects [21**]. Once confirmed in vivo, these data suggest that prevention of metastasis development through anti-inflammatory (Cox-2 axis) or additional epigenetic miR-mediated mechanisms might be a promising and highly relevant direction for translational cancer (prevention) research with ITCs. Accordingly, Powolny et al. [22] convincingly demonstrated that PEITC (3 μmol/g diet) decreased tumor incidence and tumor burden in the TRAMP transgenic mouse model for prostate cancer, and identified biomarkers for PEITC treatment by plasma proteome profiling. PEITC significantly induced expression of E-cadherin and reduced number and size of pulmonary metastases by more than 35 and 60%, respectively. Well investigated mechanisms associated with ITC action, such as inhibition of proliferation, induction of apoptosis, or inhibition of neo-angiogenesis were not affected by PEITC treatment in this model [22]. As described above, changes in miRNA expression might contribute to the observed effects.

Xiao et al. [23*] analyzed the influence of PEITC on the expression of small heterodimer partner protein (Shp, also known as NR0B2) in prostate (cancer) cell lines. Shp is a small orphan receptor that functions as a transcriptional corepressor through interaction with nuclear receptors, including retinoic X receptor, estrogen receptor, or androgen receptor. In comparison with nonmalignant prostate epithelial cells, Shp expression was reduced in all prostate cancer cell lines. This was mediated through direct interaction of Shp 3’-UTR with miR-141 that was significantly upregulated in the cancer cell lines. Overexpression of Shp decreased dihydrotestosterone (DHT)-mediated activation of androgen receptor and induction of prostate-specific antigen (PSA) in androgen receptor-positive LNCaP cells, indicating a functional interaction and repressive effect of Shp on androgen receptor signaling. PEITC treatment of LNCaP cells inhibited DHT-mediated induction of PSA. This was attributed to induced expression of Shp by reduction of miR-141 levels after PEITC treatment [23*]. Several studies found miR-141 as one of the best markers for high-risk prostate cancer with serum or plasma
levels upregulated in prostate cancer vs. healthy controls, and in metastatic prostate cancer vs. localized prostate cancer, respectively [24*]. Whether this is functionally related to downregulation of Shp or other unidentified targets is presently unknown. Notably, miR-200c (induced by SPN in [21**]) and miR-141 (reduced by PEITC in [23*]) are located in close vicinity on chromosome 12p13.31, suggesting coregulation of expression. Taken together, these data suggest that modulation of miRNA expression contributes to the cancer preventive potential of ITCs and might be relevant for prevention of metastases.

CONCLUSION

This short review summarized a recent finding on epigenetic mechanisms targeted by ITCs. The concept that ITC metabolites lead to hyperacetylation of histones generally associated with demethylation of chromatin and enhanced transcription has matured in the past year and is shown in several studies. The fact that chromatin-modifying proteins have been identified as intracellular binding targets of ITCs might contribute to the observed effects, but needs to be further investigated.

Evidence that ITCs influence DNA methylation is still limited to a few studies focussing on selected candidate genes. Genome-wide analysis of DNA methylation changes might help to understand whether the described observations are targeted events or associated with global hypomethylation, for example, through inhibition of DNMT expression. Global DNA hypomethylation is a ‘hallmark’ of cancer and associated with increased genomic instability. Conceptually, discussion is needed on whether global reduction in DNA methylation is a valid concept for cancer prevention. Rather, one would expect that prevention of DNA hypomethylation, for example, by prevention of oxidative stress as shown in hypertensive rats [13*], would be associated with prevention of cancer.

Studies applying the concept of combination chemoprevention targeting epigenetic mechanisms have made interesting findings with potential relevance for translational cancer (prevention) research.

Most exciting results have been obtained in the field of noncoding microRNAs. Recent advances in array-based and sequencing technologies have facilitated profiling of miRNA expression in tissue or circulating in serum [25] and other body fluids [24*], underlining their potential role in intracellular communication [24*] or use as biomarkers for detection of tumors or other diseases [25]. Presently it is still unknown how dietary chemopreventive agents including ITCs modulate miRNA expression, but investigation on underlying mechanisms will be an important research topic in the future.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

4. Comprehensive review on efficacy of ITCs in rodent models and overview of underlying mechanisms.
9. This study reveals a series of direct intracellular interaction partners of ITCs, including a chromatin modifier and histone proteins.
11. Critical perspective on the pathway by one of the leading scientists in cancer chemoprevention research.
13. Unbiased approach to identify genes regulated by the KEAP1/Nrf2/ARE pathway reveals novel targets.
15. Study showing that epigenetically silenced Nrf2 can be reactivated by a combination of mechanisms.
20. This study is of relevance as it demonstrated that prevention of oxidative stress leads to normalization of disease (hypertension)-associated hypomethylation.


This study describes that the combination of two chemopreventive agents with distinct epigenetic targets is more efficient. Also, re-expression of ER in hormone-receptor negative breast cancer might be of high clinical relevance.


If confirmed in vivo, this study might provide an interesting new direction for translational cancer (prevention) research targeting metastasis formation.


Ref. [23] might be of relevance for [22].

24. Hessvik NP, Sandvig K, Llorente A. Exosomal miRNAs as Biomarkers for Prostate Cancer. Front Genet 2013; 4:36. Review that summarizes the potential of circulating miRNAs as biomarkers for cancer detection or prognosis. This is of interest for monitoring the effects of chemopreventive agents.