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Cancer chemoprevention with dietary isothiocyanates mature for clinical translational research

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Inverse association between dietary intake of cruciferous vegetables and cancer risk observed in population-based case-control studies is partly attributable to structurally simple but mechanistically complex phytochemicals with an isothiocyanate (–N=C=S) functional group. Cancer protective role for dietary isothiocyanates (ITCs) is substantiated by preclinical studies in rodent models. A common feature of many naturally occurring ITCs relates to their ability to cause growth arrest and cell death selectively in cancer cells. At the same time, evidence continues to accumulate to suggest that even subtle change in chemical structure of the ITCs can have a profound effect on their activity and mechanism of action. Existing mechanistic paradigm stipulates that ITCs may not only prevent cancer initiation by altering carcinogen metabolism but also inhibit post-initiation cancer development by suppressing many processes relevant to tumor progression, including cellular proliferation, neoangiogenesis, epithelial–mesenchymal transition, and self-renewal of cancer stem cells. Moreover, the ITCs are known to suppress diverse oncogenic signaling pathways often hyperactive in human cancers (e.g. nuclear factor-κB, hormone receptors, signal transducer and activator of transcription 3) to elicit cancer chemopreventive response. However, more recent studies highlight potential adverse effect of Notch activation by ITCs on their ability to inhibit migration of cancer cells. Mechanisms underlying ITC-mediated modulation of carcinogen metabolism, growth arrest, and cell death have been reviewed extensively. This article provides a perspective on bench-cage-bedside evidence supporting cancer chemopreventive role for some of the most promising ITCs. Structure–activity relationship and mechanistic complexity in the context of cancer chemoprevention with ITCs is also highlighted.

Introduction

Social and economic burden from cancer is still quite substantial around the world despite increasing awareness of life-style risk factors (e.g. smoking) and screening efforts for early detection of the disease. Novel approaches for prevention of cancer are desirable mainly because many risk factors associated with tumor development are not easily modifiable (e.g. genetic predisposition). Chemoprevention of cancer is feasible as exemplified by selective estrogen receptor modulators and aromatase inhibitors for breast cancer risk reduction (1–3). Data accumulated over the past three decades provides compelling preclinical evidence for cancer protective effect of isothiocyanates (ITCs) derived from edible cruciferous vegetables. Despite convincing preclinical evidence, however, the progress toward clinical translation for ITCs has been rather disappointing probably due to a variety of reasons, including lack of suitably formulated agents for oral administration, regulatory issues requiring investigational new drug application submission and approval from the Federal Drug Administration, and complexities associated with primary prevention clinical trials requiring thousands of subjects and years of follow-up to draw meaningful conclusions. This article summarizes preclinical evidence for cancer preventive role for some of the most promising ITCs, including watercress constituent phenethyl isothiocyanate (PEITC), garden cress constituent benzyl isothiocyanate (BITC) and synthetic racemic analogue of broccoli constituent L-sulforaphane (D,L-sulforaphane; hereafter abbreviated as SFN) (4–6). Chemical structures of BITC, PEITC and SFN are shown in Figure 1.

The ITCs are stored as thioglucoside conjugates (commonly known as glucosinolates) in cruciferous vegetables (7,8). For example, the glucosinolate precursor of PEITC is gluconasturtiin, whereas L-sulforaphane is stored as glucoraphanin in cruciferous vegetables (8). Plant tissue damage resulting from cutting or chewing of the cruciferous vegetables releases an enzyme (myrosinase) that is responsible for conversion of the glucosinolates to corresponding ITCs (8). The ITCs can also be generated by intestinal microflora (9). Substantial amounts of glucosinolates are achievable through dietary intake of the cruciferous vegetables. For example, glucosinolate content in edible cruciferous vegetables ranges from 0.5 to 3 mg/g and one ounce of watercress is estimated to result in intake of about 37 μmol of PEITC (10).

ITCs are effective inhibitors of chemically induced cancer in experimental rodents

Wattenberg was the first to report inhibition of chemically induced cancer in experimental rodents upon PEITC and BITC administration more than 30 years ago (11). Specifically, the PEITC administration 4 hours before 7,12-dimethylbenz[*a*]anthracene administration resulted in inhibition of mammary carcinogenesis in rats (11). Since then, a number of studies from different laboratories have documented protective effect of PEITC and BITC against cancer in rodents induced by structurally diverse chemical carcinogens. In this context, contributions of Gary D. Stoner, Late Bandaru S. Reddy, Stephen S. Hecht, Fung-Lung Chung, and Paul Talalay *et al.* are noteworthy (10,12–15). Some of their seminal studies documenting ITC-mediated prevention of chemically induced cancers are summarized in Table I and exemplified later.

Stoner group showed that the rats fed a diet supplemented with 3 and 6 mmol PEITC/kg diet before (pre-initiation) as well as during treatment with the carcinogen *N*-nitrosobenzylmethylamine (post-initiation) developed significantly fewer esophageal tumors compared with rats fed a control diet (12). Lung tumorigenesis induced by the tobacco-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in rats was inhibited significantly by dietary administration of 4 and 8 mmol PEITC/kg diet (13). Gavage of PEITC inhibited azoxymethane-induced colonic aberrant crypt foci in rats providing important laboratory evidence for protection against colon cancer (15). Feeding of diet supplemented with

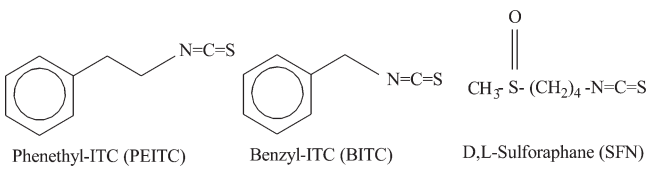

Fig. 1. Chemical structure of phenethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC), and sulforaphane (SFN).

Abbreviations: BITC; benzyl isothiocyanate; EMT, epithelial to mesenchymal transition; ITCs, isothiocyanates; PEITC, phenethyl isothiocyanate; ROS, reactive oxygen species; SFN, D,L-sulforaphane; TRAMP, transgenic adenocarcinoma of mouse prostate.

Table I. Protective effect of isothiocyanates (ITCs) against chemically-induced cancer in rodents

Compound	Activity	Carcinogen	Rodent species	Reference
PEITC	Inhibition of mammary carcinogenesis	DMBA	Female Sprague-Dawley rat	(11)
	Inhibition of esophageal tumorigenesis	NBMA	Male F344 rat	(12)
	Inhibition of lung tumorigenesis	NNK	Male F344 rat	(13)
	Inhibition of aberrant crypt foci formation	AOM	Male F344 rat	(28)
	Inhibition of colon tumor multiplicity	AOM/DSS	Male C57BL/6 mice	(16)
	Inhibition of oral squamous epithelial carcinogenesis	NBMA	Male Syrian golden hamster	(19)
	Inhibition of neoplasia of the forestomach	BaP	A/J mouse	(21)
	Inhibition of lung tumor multiplicity	BaP+NNK	A/J mouse	(22)
PEITC-NAC	Inhibition of lung tumorigenesis	BaP	A/J mouse	(18)
BITC	Inhibition of mammary carcinogenesis	DMBA	Female Sprague-Dawley rat	(11)
	Inhibition of neoplasia of the breast	DMBA	Female Sprague-Dawley rat	(20)
	Inhibition of lung tumorigenesis	BaP	A/J mouse	(21)
	Inhibition of lung tumorigenesis	BaP	Female A/J mouse	(23)
	Inhibition of neoplasia of the forestomach	DEN	Female A/J mouse	(23)
	Inhibition of intestinal carcinogenesis	MAM acetate	Female ACI/N rat	(24)
	Inhibition of lung tumorigenesis	5-MeC+DBahA	A/J mouse	(25)
	Inhibition of liver tumorigenesis	DEN	Male ACI/N rat	(26)
	Inhibition of pancreatic carcinogenesis	BOP	Male Syrian hamster	(27)
SFN	Inhibition of mammary tumorigenesis	DMBA	Female Sprague-Dawley rat	(14)
	Inhibition of colonic aberrant crypt foci	AOM	Male F344 rat	(28)
	Inhibition of neoplasia of the forestomach	BaP	Female ICR mouse	(29)
	Inhibition of skin tumorigenesis	DMBA/TPA	Female CD-1mouse	(31)

Abbreviations: PEITC, phenethyl isothiocyanate; PEITC-NAC, *N*-acetylcysteine conjugate of PEITC; BITC, benzyl isothiocyanate; SFN, sulforaphane; DMBA, 7,12-dimethylbenz[a]anthracene; NBMA, *N*-nitrosobenzylmethylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AOM, azoxymethane; DSS, dextran sodium sulfate; BaP, benzo[a]pyrene; DEN, diethylnitrosamine; MAM acetate, methylazoxymethanol acetate; 5-MeC, 5-methylchrysene; DBahA, dibenz[a,h]anthracene; BOP, *N*-nitrosobis(2-oxopropyl)amine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

0.05% PEITC before or after azoxymethane initiation resulted in lower tumor incidence, lower colon tumor multiplicities and smaller polyps, as compared with mice fed with the basal diet (16). Notably, Plate and Gallaher (17) failed to observe PEITC-mediated prevention of aberrant crypt foci in rats. Yang *et al.* (18) showed inhibition of benzo(*a*)pyrene-induced lung tumorigenesis in A/J mice by dietary *N*-acetylcysteine conjugate of PEITC administered during the post-initiation phase. PEITC administration was also shown to suppress *N*-nitrosomethylbenzylamine-induced hamster buccal pouch cancer (19). Similar to PEITC, cancer protective role for BITC has been demonstrated in a number of chemically induced rodent cancer models, including 7,12-dimethylbenz[*a*] anthracene-induced breast cancer and benzo[*a*]pyrene-induced forestomach cancer and pulmonary adenoma using rats or mice (20–23), diethylnitrosamine-induced forestomach tumor in mice (23), methylazoxymethanol acetate-induced intestinal carcinogenesis in rats (24), 5-methylchrysene and dibenz[*a*,*h*]anthracene-induced lung cancer in mice (25), diethylnitrosamine-induced liver cancer in rats (26), and *N*-nitrosobis(2-oxopropyl)amine-induced pancreatic atypical hyperplasia and adenocarcinoma in hamsters (27).

Talalay *et al.* are credited with sparking research interest in SFN by demonstrating preventive activity against 9,10-dimethyl-1,2-b enzanthracene-induced breast cancer in rats (14). Subsequently, chemopreventive response to SFN was extended to other chemical carcinogens. For example, both pre- and post-initiation administration of SFN resulted in suppression of colonic aberrant crypt foci in rats induced by azoxymethane (28). However, SFN–*N*-acetylcysteine conjugate exhibited activity only against post-initiation cancer development (28). SFN-mediated inhibition of benzo[*a*]pyrene-induced forestomach cancer in mice has also been demonstrated (29). SFN and its *N*-acetylcysteine conjugate inhibited malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice (30). SFN treatment was shown to prevent chemically induced skin cancer during the stage of promotion (31). It is important to mention that some studies have used the naturally occurring L-isomer whereas others have utilized synthetic racemic compound in these studies (14,28–31). Nevertheless, it is now obvious that ITCs can offer protection against cancer in experimental rodents induced by structurally diverse chemical carcinogens.

Bladder carcinogenesis/promotion in rats is concerning for some ITCs

A few studies, albeit inconsistently, have suggested that some ITCs might act as a bladder carcinogen or promote chemically induced bladder tumorigenesis at least in rats. Using a rather complex multiorgan carcinogenesis model in rats involving a single intraperitoneal injection of diethylnitrosamine, 4 intraperitoneal injections of *N*-methylnitrosourea as well as *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine administration in the drinking water during the first 2 weeks followed by 4 subcutaneous injections of dimethylhydrazine as well as 2,2'-dihydroxy-di-*n*-propylnitrosamine in the drinking water over 2 weeks, PEITC administration lowered the induction of esophageal hyperplasia, kidney atypical tubules, and liver glutathione *S*-transferase placental form-positive foci when given during the initiation period but enhanced the development of liver glutathione *S*-transferase placental form-positive foci and urinary bladder tumors if administered in the post-initiation period (32). Additional work from this same group showed that PEITC-induced papillary or nodular hyperplasia, dysplasia, and transitional cell carcinoma in a dose-dependent manner but only after initiation with diethylnitrosamine and *N*-butyl-*N*-(4-hydrozybutyl)nitrosamine (33). Whether PEITC alone acts as a carcinogen in the urinary bladder is also controversial. Some studies indicate that PEITC can promote proliferation in normal looking epithelium leading to dysplasia (34). Other studies indicated that PEITC treatment had only limited potential to initiate abnormal growth and did not effectively induce irreversible lesion in urinary bladder (35). Similar inconsistencies are discernible for BITC in the context of bladder cancer in rats. In experiments designed to determine the post-initiation effect of BITC against hepatocarcinogenesis and urinary bladder carcinogenesis, rats were pretreated with diethylnitrosamine and *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine prior to dietary BITC administration (36). Incidence of papillary or nodular hyperplasia and carcinoma were significantly elevated in the BITC treatment group (36). Interestingly, BITC potently inhibited induction of these lesions when given simultaneously with the carcinogen (37). In a follow-up study, simultaneous treatment with BITC and a lower dose of the same carcinogen did not inhibit, but rather enhanced rat urinary bladder carcinogenesis (38).

These observations will undoubtedly pose difficulties in regulatory approval of PEITC or BITC as a potential chemopreventive agent for long-term administration in high-risk but otherwise normal healthy subjects. At the same time, a few arguments lend support for long-term administration of these agents for cancer chemoprevention purpose. First, bladder carcinogenesis or promotion of chemically induced bladder cancer by PEITC or BITC has not been reported in any other rodent species (e.g. mouse). Second, PEITC/BITC-containing cruciferous vegetables are consumed by humans on a daily basis in some cultures, yet epidemiological studies have suggested an inverse association between intake of these vegetables and bladder cancer risk and survival (39–41). It is possible that rats are unusually sensitive to bladder carcinogenesis by PEITC and BITC. Finally, human relevance of the multiorgan rat carcinogenesis models employed to demonstrate bladder tumor promoting effects of PEITC and BITC (32,36) is unclear if not questionable. Notably, at least two studies have documented clastogenic effects of BITC (42,43). Furthermore, BITC treatment exhibited genotoxic effects in HepG2 cells but substantially weaker effects were obtained *in vivo* (44). Clearly, toxicological evaluations after long-term administration of PEITC and BITC in different species are needed to advance these agents in clinical translational studies.

Bladder carcinogenesis or promotion of chemically induced bladder cancer has not been reported for SFN. In fact, a recent study showed that SFN administration inhibited DNA adduct formation induced by a bladder carcinogen, 4-aminobiphenyl, in a NF-E2 related factor-2-dependent manner in bladder cells and tissues (45). Furthermore, dietary administration of a freeze-dried aqueous extract of broccoli sprouts, which is a rich source of glucoraphanin, conferred significant and dose-dependent protection against bladder cancer development in rats induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (46). It is important to note that the broccoli sprout extract itself did not cause any histologic changes in the bladder (46).

ITCs prevent oncogene-driven cancer development in transgenic mice

Availability of transgenic mouse models in recent years has enabled determination of chemopreventive efficacy of the ITCs against spontaneous cancer development. Key studies documenting cancer chemoprevention by ITCs in transgenic mouse models are summarized in Table II. For example, the $Apc^{Min/+}$ mice fed a diet supplemented with 0.05% PEITC for 3 weeks developed significantly less (31.7% reduction) and smaller polyps than those fed basal diet (47). Dietary feeding of 8 mmol PEITC/kg diet to polyoma middle-T antigen transgenic mice resulted in smaller mammary cancer lesions, although there was no effect on lung metastasis or survival (48). Feeding of a diet supplemented with 0.05% PEITC alone or 0.025% PEITC in combination with 1% curcumin, a constituent of turmeric, significantly decreased incidence of prostate tumor in transgenic adenocarcinoma of mouse prostate (TRAMP) mice (49). Recent studies from our laboratory have revealed that administration of 3 mmol PEITC/kg diet decreases incidence as well as burden (affected area) of poorly differentiated tumors in the dorsolateral prostate of TRAMP mice (50). Dietary administration of 3 mmol BITC/kg diet for 25 weeks markedly suppressed the incidence and/or burden of mammary hyperplasia and carcinoma in female MMTV-*neu* transgenic mice without causing weight loss or affecting neu protein level (51). The BITC-mediated prevention of mammary carcinogenesis in MMTV-*neu* mice was shown to be associated with T-cell infiltration and induction of E-cadherin (51). Mammary cancer in MMTV-*neu* mice is also suppressed by dietary administration of 3 mmol PEITC/kg diet (S.V.Singh *et al*., unpublished results). Long-term administration of SFN in the diet resulted in suppression of tumor development in Apc^{min} mice (52). Dietary administration of SFN (300 and 600 ppm) for 3 weeks to $Apc^{Min/+}$ mice resulted in suppression of polyps in the small intestine in a dose-dependent manner (53). Oral gavage of 6 μmol SFN thrice per week beginning at 6 weeks of age significantly inhibited prostate intraepithelial neoplasia and pulmonary metastasis in TRAMP mice (54). Consistent with these results, 8-week-old TRAMP mice fed with 240 mg of broccoli sprouts/mouse/day exhibited a significant retardation of prostate tumor growth in another study (55).

ITCs inhibit processes relevant to cancer progression

Unregulated cellular proliferation, evasion of apoptosis, and neo-angiogenesis (formation of new blood vessels) are hallmarks of cancer progression. Interestingly, all these processes are sensitive to inhibition by ITCs. For example, PEITC, BITC and SFN inhibit cellular proliferation in association with G_2/M phase cell-cycle arrest and apoptosis induction. Mechanistic complexity underlying growth arrest and cell death by PEITC is exemplified in Figure 2. Hasegawa *et al*. (56) were the first to report G_2/M phase cell-cycle arrest upon treatment with PEITC and BITC in HeLa cells. Except for a few reports, most studies have shown G_2/M phase cell-cycle arrest in cancer cells upon treatment with PEITC and BITC (57–59). For example, G_2/M phase cell-cycle arrest after treatment with BITC was shown in leukemia (60), pancreatic (61) and breast cancer cells (62). However, a few studies have shown arrest in other phases of the cell cycle upon treatment

Table II. Chemopreventive efficacy of isothiocyanates (ITCs) in transgenic mouse models

Agent	Transgenic mouse model	Experimental protocol	Effect	Reference
PEITC	$Apc^{Min/+}$ mouse model of gastrointestinal cancer	Dietary administration of 0.05% PEITC	Inhibition of intestinal polyp development and reduced intestinal tumor size	(47)
	Polyoma middle-T antigen (PyMT) transgenic mouse model of breast cancer	Dietary feeding of 8 mmol PEITC/kg	Reduced size of mammary cancer lesions	(48)
	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Dietary feeding of 0.05% PEITC alone or 0.025% PEITC + 1% curcumin	Inhibition of prostate tumor incidence	(49)
	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Dietary administration of 3 mmol PEITC/kg	Inhibition of incidence and burden of poorly-differentiated prostate tumor	(50)
BITC	MMTV-*neu* transgenic mouse model of breast cancer	Dietary administration of 3 mmol BITC/kg	Suppression of incidence and/or burden of hyperplasia and carcinoma	(51)
SFN	Apc^{Min} mouse model of gastrointestinal cancer	Dietary feeding of ~6 μmol SFN/day	Suppression of polyp formation	(52)
	$Apc^{Min/+}$ mouse model of gastrointestinal cancer	Dietary feeding of 300 or 600 ppm of SFN	Suppression of polyps in the small intestine	(53)
	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Oral gavage of 6 μmol SFN thrice a week	Inhibition of prostate intraepithelial neoplasia and pulmonary metastasis	(54)
	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Feeding with 240 mg broccoli sprouts/mouse/day	Inhibition of prostate tumor growth	(55)

Abbreviations: PEITC, Phenethyl isothiocyanate; BITC, Benzyl isothiocyanate; SFN, Sulforaphane

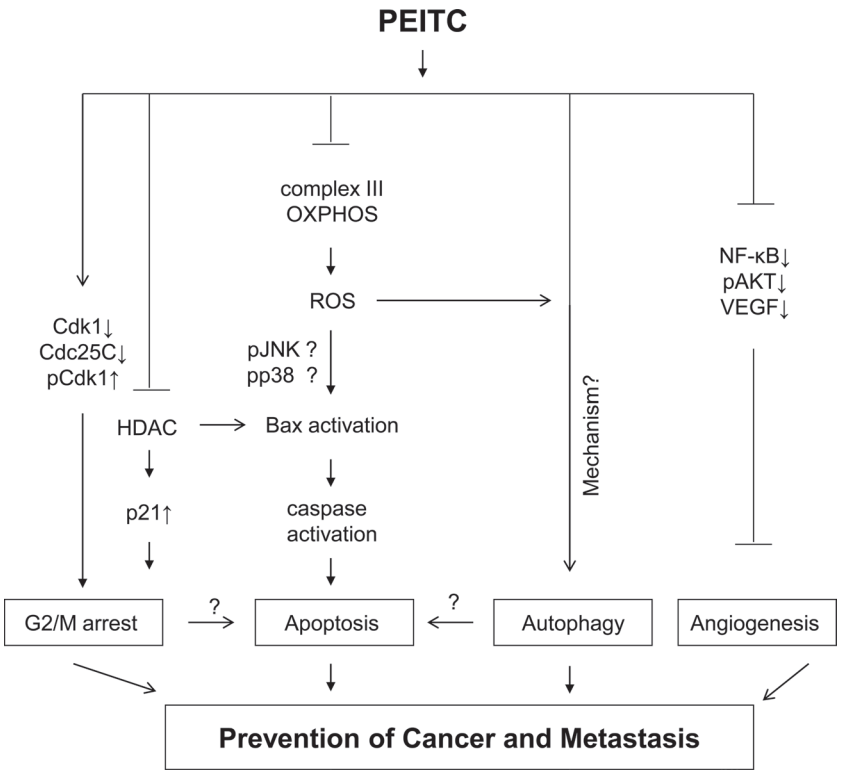

Fig. 2. Mechanisms underlying inhibition of post-initiation cancer development by PEITC. Post-initiation cancer chemoprevention by PEITC probably involves (i) growth arrest at G_2/M phase of the cell cycle due to downregulation of cyclin-dependent kinase 1 (Cdk1) and cell division cycle 25C (Cdc25C) leading to accumulation of tyrosine-15 phosphorylated (inactive) Cdk1 (57); inhibition of histone deacetylase (HDAC) leading to induction of p21 may also contribute to cell cycle arrest by PEITC and SFN (6), (ii) apoptosis mediated by reactive oxygen species (ROS) resulting from inhibition of complex III of the mitochondrial respiratory chain (81); the PEITC-induced ROS results in Bax activation leading to caspase activation and ultimately cell death (81); the ROS-mediated Bax activation may be mediated by activation of c-Jun-N-terminal kinases (JNK) and/or p38 mitogen activated protein kinase similar to that reported for benzyl isothiocyanate (80), (iii) induction of autophagic death that is partly dependent on ROS (100); mechanism underlying PEITC-induced autophagy is still unclear, and (iv) inhibition of angiogenesis due to suppression of nuclear factor-κB, phosphorylated (active) AKT, and vascular endothelial growth factor (VEGF) signaling (87,112). This illustration does not fully capture every mechanistic alteration resulting from PEITC exposure but signifies mechanistic complexity by which PEITC may prevent post-initiation cancer development.

with BITC (63,64). Similar discrepancies are also found for SFN (65–68). For example, SFN treatment was shown to cause G_2/M phase cell-cycle arrest in HT29 colon cancer cells (65) and androgen-independent PC-3 prostate cancer cells (67), whereas androgen-responsive LNCaP prostate cancer cells exposed to SFN were arrested in both S and G_2/M phases of the cell cycle (68). Mechanisms by which ITCs cause cell-cycle arrest have been reviewed elsewhere (4–6). As an example, using PC-3 cells as a model we demonstrated important contribution of checkpoint kinase-2 activation leading to phosphorylation and cytoplasmic sequestration of cell division cycle 25C in SFN-mediated G_2/M phase cell-cycle arrest (67).

One of the unique properties of ITCs is their ability to selectively cause apoptosis in cancer cells. Differential sensitivity of cancer cells *versus* normal epithelial cells to apoptosis induction has been noted for PEITC (4,69), BITC (5,62) and SFN (70). Research over the past decade reveals that the molecular circuitry of apoptosis induction by ITCs is complex and utilizes a wide range of molecular mechanisms, including alterations in Bcl-2 family protein expression, activation of mitogen-activated protein kinases, suppression of oncogenic signaling pathways and activation of caspases (reviewed in refs. 4–6,71). Yu *et al.* (72) were the first to show apoptosis induction by PEITC in HeLa cells. During the same time period, Huang *et al.* (73) used mouse embryonic fibroblasts to demonstrate a critical role for p53 in regulation of PEITC-induced apoptosis. This association was found to be unique to the mouse embryonic fibroblasts because PEITC was later shown to cause apoptosis in p53-deficient cancer cells (74). Lack of p53 involvement in apoptosis induction by BITC has also been demonstrated (75). Interestingly some ITCs selectively deplete mutant p53, but not the wild-type p53, *via* a transcription-independent mechanism (76). Direct p53 binding followed by conformational change is implicated in depletion of mutant p53 by some ITCs (76). Mechanistic complexity of apoptosis induction by PEITC, BITC and SFN has been reviewed previously (4–6,71) but production of reactive oxygen species appears to be a common link in apoptosis induction by PEITC, BITC and SFN (77–82). Interestingly, normal cells are resistant to ROS production by ITCs (78,81). The mechanism of ROS production and signal transduction downstream of ROS production in execution of apoptosis by PEITC, BITC and SFN involves inhibition of mitochondrial respiratory chain and activation of multidomain proapoptotic protein Bax, respectively (80–82). The mechanism underlying differential sensitivity of cancer cells and normal cells to apoptosis induction by ITCs is still unclear but PEITC treatment has been shown to differentially alter expression of oxidative stress and antioxidant defense genes in PC-3 (a prostate cancer cell line) versus PrEC cells (a normal prostate epithelial cell line) (83). A role for the adapter protein $p66^{Shc}$ has also been established in ROS production and apoptosis induction by PEITC as well as SFN (84,85).

Neoangiogenesis is critical not only for tumor growth but also for metastatic spread (86). Several groups have thoroughly investigated anti-angiogenic effect of ITCs. For example, PEITC treatment decreased expression of vascular endothelial growth factor and inhibited capillary-like tube structure formation (a measure of neoangiogenesis) and migration in human umbilical vain endothelial cells (87). Furthermore, the PEITC treatment inhibited angiogenesis *ex vivo* as revealed by chicken egg chorioallantoic membrane assay (87). PEITC is an effective inhibitor of hypoxia inducible factor (88),

a proangiogenic transcription factor. Because angiogenesis plays an important role in metastasis, a previous study from our group determined the effect of PEITC administration on incidence and multiplicity of pulmonary metastasis in TRAMP model (50). Overall incidence of pulmonary metastasis did not differ between the control and the PEITC-treated mice but the number of lung metastasis per mouse in the mice fed PEITC-supplemented diet was about 38% lower than that in the mice fed control diet (50). In MDA-MB-231 xenografts, analysis of the vasculature in the tumors from BITC-treated mice indicated smaller vessel area compared with control tumors based on immunohistochemistry for angiogenesis marker CD31 (89). The BITC-mediated inhibition of angiogenesis *in vivo* correlated with downregulation of vascular endothelial growth factor receptor 2 protein levels in the tumor (89). Oral BITC treatment reduced hemoglobin content, CD31 and vascular endothelial growth factor expression *in vivo* (90). The BITC-mediated inhibition of neoangiogenesis in rat aorta and chicken-chorioallantoic membrane models was also shown (91). The inhibitory effect of SFN on endothelial cell function essential for angiogenesis were shown using HMEC-1, an immortalized human microvascular endothelial cell line (92). The SFN treatment suppressed angiogenesis and disrupted endothelial mitotic progression and microtubule polymerization (93). Anti-angiogenic effect for ITCs has been reviewed by Cavell *et al.* (94). In summary, it is reasonable to conclude that inhibition of post-initiation cancer development by ITCs is probably achieved by suppression of multiple processes relevant to cancer progression.

Functional significance of autophagy induction by PEITC/BITC versus SFN

Autophagy is an evolutionarily conserved process for bulk degradation of macromolecules including organelles (e.g. mitochondria) (95). Autophagy is considered a valid cancer chemotherapeutic target (95,96). Our laboratory was the first to document autophagy induction by SFN in prostate cancer cells (97). Autophagy induction by SFN was since then confirmed by other investigators in different cellular systems (98,99). Even though autophagy induction is not unique to SFN as this process is activated upon treatment of cancer cells with PEITC (100) and BITC (101) and the autophagic response to all three agents is partially linked to ROS production (81,82,101), outcome of this cellular response is strikingly different for PEITC and BITC versus SFN (97,100,101). Autophagy serves to inhibit apoptosis induction by SFN by preventing release of cytochrome *c* from mitochondria to the cytosol (97). To the contrary, autophagy induction contributes to cell death by PEITC and BITC (100,101). Previous studies from our laboratory have also provided *in vivo* correlative evidence for autophagy induction by PEITC and BITC (50,100,101). Similar to apoptosis, normal epithelial cells are significantly more resistant to induction of autophagy by PEITC and BITC compared with cancer cells (100,101). Mechanism underlying autophagy induction by PEITC or SFN is still unresolved, but BITC-mediated autophagy is associated with increased acetylation of FoxO1 (101).

ITCs inhibit self-renewal of cancer stem cells

Evidence continues to accumulate to suggest that resistance of cancer stem cells to conventional therapy (e.g. chemotherapy, hormonal therapy, radiotherapy) is a major cause of disease recurrence (102,103). It was shown recently that SFN can suppress self-renewal of breast cancer stem cells characterized by a significant decrease in aldehyde dehydrogenase 1-positive cell population and reduction in the size and number of primary mammospheres (104). The SFN-mediated inhibition of self-renewal of breast cancer stem cells correlated with suppression of Wnt/β-catenin pathway (104). Furthermore, daily injection with 50 mg/kg SFN for 2 weeks reduced aldehyde dehydrogenase 1-positive cells by >50% in a breast cancer xenograft model (104). Unpublished studies from our laboratory also indicate inhibitory effect of BITC on self-renewal of breast cancer stem cells (S.V.Singh, unpublished results). Efficacy of PEITC against cancer stem cells and that of BITC or SFN against cancer stem cells of other organs is yet to be determined.

BITC inhibits epithelial to mesenchymal transition in breast cancer cells

Using human breast cancer cells (MDA-MB-231 and MCF-7) as a model, we have demonstrated previously that BITC inhibits epithelial to mesenchymal transition (EMT) (105). The EMT is essential for normal physiological processes such as embryonic development, tissue remodeling and wound healing (106). During EMT, epithelial phenotype characterized by tight cell–cell junctions and polarity changes to a mesenchymal phenotype typified by disruption of the cell–cell contact with conversion to fibroblastic morphology and increased motility (106). The EMT is implicated in progression of cancers to invasive state (106). The BITC-mediated inhibition of EMT in breast cancer cells was characterized by upregulation of E-cadherin and downregulation of mesenchymal markers, including vimentin and fibronectin (105). Our observations of EMT inhibition by BITC have since been confirmed by other investigators (107). The mechanism by which BITC inhibits EMT is still unresolved, but the BITC-mediated growth retardation of MDA-MB-231 xenograft *in vivo* is associated with induction of E-cadherin and suppression of vimentin and fibronectin protein levels in the tumor (105). Studies are needed to determine if anti-EMT effect is unique to BITC.

Lack of target-specificity is probably beneficial for cancer chemoprevention by ITCs

Lack of target-specificity is a frequently voiced sentiment for naturally occurring chemopreventive agents, and ITCs are no exception to this potential criticism. In our view, ability to target multiple pathways is a desirable attribute for chemopreventive agents because pathogenesis of cancer is complex often characterized by deregulation of multiple checkpoints and activation of several oncogenic pathways. Agents selective against a single pathway/molecule may have limited clinical utility as exemplified by the estrogen receptor antagonists (1,2). An overall mechanistic model emerging from research over the past two decades stipulates that ITCs, including PEITC, BITC and SFN, have the ability to not only inhibit cancer initiation by decreasing carcinogen activation and/or increasing detoxification of the activated carcinogenic intermediates but also prevent post-initiation cancer development by affecting a variety of processes relevant to cancer progression. Alteration of carcinogen metabolism as a likely mechanism for the ITC-mediated inhibition of cancer initiation has been reviewed extensively elsewhere (71,108–110), but some of the notable mechanisms potentially contributing to post-initiation cancer chemoprevention by ITCs include: (i) inhibition of histone deacetylase (111); (ii) inhibition of oncogenic transcription factors (e.g. nuclear factor-κB, signal transducer and activator of transcription 3, androgen receptor and estrogen receptor-α) (61,112–116); (iii) protein binding (117) and (iv) inhibition of cap-dependent translation (118). However, relative contribution of these mechanisms to cancer chemoprevention by ITCs is hard to predict.

Even a subtle change in the ITC structure can have a profound impact on its activity

A potential misperception about ITCs is that they share common mechanism of action. On one hand, this argument has some validity considering most ITCs are inducers of phase 2 enzymes contributing to their pre-initiation chemopreventive effects (4-6,10,108). At the same time, examples exist to illustrate that even subtle difference in the ITC structure can translate into striking mechanistic divergence. For example, we have shown recently that apoptosis induction by PEITC is mediated by Bim in MCF-7 and MDA-MB-231 human breast cancer cells (119). Surprisingly, Bim is dispensable for proapoptotic response to BITC in the same cell lines (120). Autophagy is another example

to highlight mechanistic differences between ITCs (97,100,101). Noticeable differences in efficacy of ITCs for prevention of chemically induced as well as spontaneous cancers in rodents have also been reported. For instance, SFN and PEITC seem to inhibit different stages of prostate cancer development in the TRAMP model (50,54). Although SFN treatment inhibits incidence of prostatic intraepithelial neoplasia and well-differentiated cancer (54), PEITC is an effective suppressor of poorly differentiated prostate cancer in TRAMP model (50). Interestingly, prostate cancer prevention by SFN in TRAMP model is associated with increased lytic activity of natural killer cells (54). On the other hand, PEITC administration has no effect on activity of natural killer cells (S.V.Singh, unpublished results).

Biomarkers of ITC exposure/response

Successful clinical realization of a chemopreventive strategy depends on systematic investigations beginning with identification of promising agents and characterization of their mechanisms of action to animal studies focusing not only on bioavailability, safety and efficacy assessments but also on discovery of biomarker(s) associated with exposure and response prior to translation in humans with a pilot biomarker modulation trial in a smaller cohort followed by larger trials with cancer incidence as the primary end point. Biomarker(s) of tissue exposure and/or response are critical for cancer chemopreventive agents because clinical trials with cancer incidence as the primary end point are expensive and laborious requiring years of follow-up and thousands of high-risk subjects. Recent studies have identified some biomarkers potentially useful in future clinical investigations of ITCs. Our own studies have utilized two-dimensional gel electrophoresis followed by mass spectrometry to identify plasma clusterin as a potential biomarker of PEITC exposure and possibly response in TRAMP model (50). Clusterin (also known as apolipoprotein J and testosterone-repressed prostate message-2) is a highly conserved protein expressed in a variety of tissues, secreted in blood, and involved in regulation of apoptosis, cell adhesion, cell-cycle, and DNA repair (121,122). Increased levels of clusterin have been reported in several malignancies including breast, colon, lung, and prostate cancer (121). Moreover, expression of clusterin correlated with Gleason score in prostate cancer patients (122). Future clinical trials will test whether clusterin is a viable biomarker to assess tissue exposure and/or response to PEITC. Stable reaction products with albumin and hemoglobin as biomarkers to monitor ITC exposure in humans have also been identified (123,124). For instance, blood samples collected from a normal healthy volunteer 1 day after ingestion of garden cress (60 g), watercress (100 g) and broccoli (300 g) revealed presence of PEITC-lysine adducts in both albumin and hemoglobin (123).

ITCs are chemotherapy sensitizers

Evidence exists to suggest that ITCs may act as sensitizers of chemotherapeutic agents. Sublethal doses of PEITC sensitized Fas-resistant T24 bladder carcinoma cell line and Bcl-2 overexpressing Jurkat T cells to Fas-mediated apoptosis (125). Inhibition of P-glycoprotein and multidrug-resistance protein 1-mediated efflux of daunomycin, which is a major mechanism of resistance for some anticancer agents, by PEITC has been described (126,127). The PEITC caused sensitization of PC-3 and HeLa cells to adriamycin and etoposide-induced apoptosis by downregulation of protein kinase C and inhibition of telomerase activity (128,129). The PEITC and BITC treatments resulted in sensitization of non-small cell lung cancer cell line NCI-H596 to cisplatin independent of cellular platinum accumulation or DNA platination (130). Studies from our laboratory have revealed that pharmacologic concentrations of PEITC augment Docetaxel-induced apoptosis in PC-3 and DU145 human prostate cancer cells in association with suppression of Bcl-2 and XIAP protein levels and induction of Bax and Bak (131). Pretreatment with BITC resulted in sensitization of BxPC-3 pancreatic cancer cells to gamma-radiation induced cell-cycle arrest and apoptosis due to inhibition of nuclear factor-κB and activation of p38 mitogen-activated protein kinase (132). The BITC treatment increased sensitivity of MIAPaCa-2 and PANC-1 pancreatic cancer cells to X-ray in association with increased apoptosis, suppression of X-linked inhibitor of apoptosis protein and increase in apoptosis protease activating factor-1 (133). The BITC treatment resulted in sensitization of a panel of pancreatic cancer cells to TRAIL-induced apoptosis due to dual activation of extrinsic and intrinsic pathways (134). The SFN potentiated effects of chemotherapy drugs (e.g. cisplatin) on inhibition of clonogenicity and spheroid formation and aldehyde dehydrogenase 1 activity along with Notch-1 and c-Rel expression in pancreatic and prostate cancer cells (135). The SFN and doxorubicin combination reversed resistance in mouse fibroblasts with $p53^{Ser220}$ mutation (136).

The *in vivo* relevance of most of these predominantly cellular findings is still unclear, but we have shown previously that PEITC–Docetaxel combination is markedly more efficacious against PC-3 xenograft *in vivo* compared with PEITC or Docetaxel alone (131). The SFN and chemotherapy drug combination was most effective and totally abolished growth of cancer stem cell xenografts and tumor-initiating potential (135). Similar *in vivo* studies with other ITC-chemotherapy drugs are necessary to spark interest among clinicians to test such combinations in cancer patients.

Some ITCs activate Notch signaling in cancer cells and normal epithelial cells

Recent studies from our laboratory have demonstrated that PEITC treatment increases cleavage (activation) of Notch1 and Notch2 in prostate cancer cells leading to transcriptional activation of Notch (137). Notch pathway is implicated in tumorigenesis, maintenance of mesenchymal phenotype and self-renewal of cancer stem cells (138–140). The PEITC-mediated activation of Notch is not selective for cancer cells as normal epithelial cells (PrEC) are also sensitive to Notch1 and Notch2 activation by PEITC (137). However, Notch activation may be a double-edge sword in the context of cancer chemoprevention with these ITC compounds. On one hand, PEITC-induced apoptosis in LNCaP and PC-3 cells was attenuated by RNA interference of Notch2, but not Notch1 (137). On the other hand, inhibition of PC-3 and LNCaP cell migration resulting from PEITC exposure was significantly augmented by knockdown of Notch2 as well as pharmacological inhibition of Notch1 activation (137). Further studies are needed to determine whether Notch1 and Notch2 activation by PEITC is unique to prostate cancer cells, and if Notch activation affects cancer chemopreventive response to PEITC *in vivo*. Activation of Notch1, Notch2, and Notch4 upon treatment with BITC has been observed in human breast cancer cells (141). The BITC-mediated inhibition of breast cancer cell migration was significantly augmented by RNA interference of Notch2, but not Notch1 or Notch4 (141). Once again these observations underscore mechanistic differences between structurally related ITC compounds.

miRNAs targeted by BITC

The miRNA function as either oncogenes or tumor suppressor and can target multiple genes (142). Recent studies have shown that BITC treatment alters the expression of miRNA-221 and miRNA-375 to switch hyperproliferative cancer cells to a hypoproliferative state in pancreatic adenocarcinoma cells (143). The miRNA-221 and miRNA-375 are abnormally expressed in pancreatic cancer patients (144). Ectopic expression of miRNA-375 and silencing of miRNA-221 sensitized cells to antiproliferative effect of BITC (143). Additional work is needed to determine if other ITCs target miRNA as well as to identify other potential miRNA targeted by ITCs.

Human studies are limited to raw cruciferous vegetables or their extracts

Human studies on biological effects of pure ITC compounds are still lacking, but a few studies have attempted to determine the effects

of raw cruciferous vegetables or their extracts on certain biological parameters (145–149). For example, consumption of 2 ounce (56.8 g) of watercress at each meal for 3 days was shown to inhibit oxidative metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in smokers (145). Consistent with cellular observations (118), phosphorylation of 4E-BP1 was significantly reduced 6 and 8 hours after ingestion of 80 g watercress in peripheral blood cells from four participants (146). Another randomized and placebo-controlled trial performed in Qidong, China, utilizing a beverage infused with 3-day-old broccoli sprouts showed an inverse association between excretion of dithiocarbamates and urinary aflatoxin-DNA adducts (148). Ingestion of 68 g of broccoli sprouts by humans resulted in a significant decrease in histone deacetylase activity in peripheral blood mononuclear cells (149).

Concluding remarks and future directions

Discovery of cancer chemopreventive potential of ITCs more than 30 years ago sparked tremendous research interest focusing on pharmacokinetic, efficacy, and mechanistic characterization of these compounds. Notably, emerging technologies and research tools (e.g. RNA interference, microarray, proteomics, etc.) have been appropriately utilized to study the mechanism by which ITCs may prevent cancer. Clinical investigation of ITCs for cancer chemoprevention seems more plausible today mainly because of knowledge acquired in the past few decades. However, a few lingering hurdles in clinical development of ITCs are noteworthy. First, formulations of pure ITCs suitable for clinical investigations are not yet available. Second, clinical trial designs must consider rapid clearance of ITCs as corresponding mercapturic acids; a single daily administration schedule may not be effective. Finally, the question of whether PEITC and BITC are promoters of bladder cancer requires further investigation because this may turn out to be a major obstacle in long-term usage of ITCs for cancer prevention in high-risk subjects.

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