

# Cruciferous Vegetables, Isothiocyanates, and Prevention of Bladder Cancer

Omkara L. Veeranki · Arup Bhattacharya · Li Tang · James R. Marshall · Yuesheng Zhang

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**Abstract** Approximately 80 % of human bladder cancers (BC) are non-muscle invasive when first diagnosed and are usually treated by transurethral tumor resection. But 50–80 % of patients experience cancer recurrence. Agents for prevention of primary BC have yet to be identified. Existing prophylactics against BC recurrence, e.g., Bacillus Calmette-Guerin (BCG), have limited efficacy and utility; they engender significant side effects and require urethral catheterization. Many cruciferous vegetables, rich sources of isothiocyanates (ITCs), are commonly consumed by humans. Many ITCs possess promising chemopreventive activities against BC and its recurrence. Moreover, orally ingested ITCs are selectively delivered to the bladder via urinary excretion. This review is focused on urinary delivery of ITCs to the bladder, their cellular uptake, their chemopreventive activities in preclinical and epidemiological studies that are particularly relevant to prevention of BC recurrence and progression, and their chemopreventive mechanisms in BC cells and tissues.

**Keywords** Bladder cancer · Isothiocyanate · Cruciferous vegetable · Chemoprevention

## Introduction

Bladder cancer (BC) is currently the fourth most common malignancy in men and the twelfth most common malignancy in women, with estimated new cases of 56,390 in men and 18,300 in women and estimated deaths of 11,179 in men and 4,410 in

women in 2014 in the USA alone [1]. Cigarette smoking (mainly exposure to aromatic amines) accounts for approximately 50 % of BC in men and women [2]. Other important risk factors include occupational exposure to aromatic amines in aluminum, leather, rubber, textile, paint and dye industries, and exposure to arsenic-laced water [3]. BC originates primarily in the transitional cell epithelium (urothelial epithelium) that lines the inner surface of the bladder and is directly exposed to urine, which is also known as transitional cell carcinoma (TCC) [4]. Approximately 75 % of newly diagnosed BC are non-muscle invasive (NMIBC), also known as superficial BC; these are typically treated with endoscopic transurethral resection (TUR), which may be followed by an intravesical therapy, depending on the stage and grade of the cancer [5]. Risk of recurrence is 50–60 % for grades 1 and 2 tumors and 80 % for grade 3 tumors [6, 7], with a median time to first recurrence of 2.7 years [8]. Recurrence of NMIBC occurs due to at least four reasons: incomplete resection, tumor cell reimplantation, growth of microscopic tumors, and new tumor formation [9]. Approximately 20 % of NMIBC progress to muscle invasive stage [10, 11]. Prognosis is poor once muscle invasion or distant metastasis has occurred. Given the high risk of recurrence and disease progression, careful surveillance after cancer removal by TUR via cystoscopy is currently a standard clinical practice; this makes BC one of the most expensive malignancies to treat per patient in the USA [12]. Intravesical therapies with Bacillus Calmette-Guerin (BCG) or chemotherapeutic agents (e.g., mitomycin C), delivered via a urethral catheter, are used to prevent or delay recurrence after TUR [13]. Although BCG has been more effective than other agents, 20–40 % of patients fail to respond [14]. Moreover, besides requiring transurethral manipulations for drug administration, the current preventive therapies are often associated with poor tolerance and high incidence of local adverse effects [15].

Many naturally occurring isothiocyanates (ITCs), including allyl isothiocyanate (AITC), benzyl isothiocyanate

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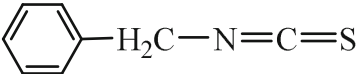
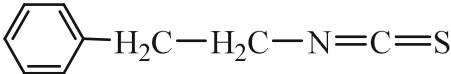
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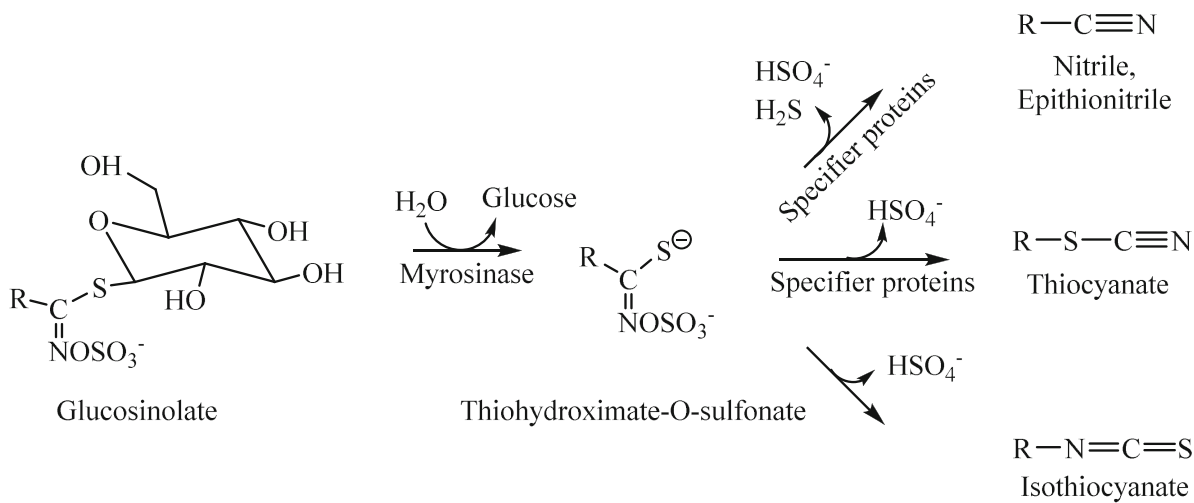
(BITC), erucin (ECN), phenethyl isothiocyanate (PEITC), and sulforaphane (SF) (see Table 1 for their chemical structures) have shown promising cancer chemopreventive activities [16–22]. The chemopreventive mechanisms of ITCs include modulation of cancer-related proteins involved in

inflammation, cell cycle arrest, cell death, angiogenesis, invasion, and metastasis along with modulation of carcinogen-metabolizing enzymes [23]. BITC, PEITC, and SF also target cancer stem cells [24–27]. ITCs inhibit the development of carcinogen-induced cancers in rodent organs, including the

**Table 1** Cruciferous vegetables, their most abundant ITCs, and conversion of glucosinolates to ITCs

| Cruciferous vegetables                  | Derived ITC | Chemical structure of ITC  |
|---|-------------|--|
| Cabbage, Horseradish<br>Mustard, Wasabi | AITC        | $\text{H}_2\text{C}=\text{HC}-\text{H}_2\text{C}-\text{N}=\text{C}=\text{S}$                                   |
| Garden cress<br>Indian cress            | BITC        |                              |
| Broccoli<br>Arugula                     | ECN         | $\text{H}_3\text{C}-\text{S}-(\text{H}_2\text{C})_4-\text{N}=\text{C}=\text{S}$                                |
| Watercress                              | PEITC       |                            |
| Broccoli<br>Broccoli sprouts            | SF          | $\text{H}_3\text{C}-\overset{\text{O}}{\parallel}{\text{S}}-(\text{H}_2\text{C})_4-\text{N}=\text{C}=\text{S}$ |

Myrosinase-catalyzed hydrolysis of glucosinolates



bladder, colon, esophagus, liver, lung, mammary gland, pancreas, skin, and small intestine [28–30]. In xenograft cancer models, AITC, BITC, PEITC, and SF inhibit BC [31, 32], breast cancer [33, 34], osteosarcoma [35], pancreatic cancer [36], or prostate cancer [37, 38]. PEITC and SF also inhibit colon cancer development induced by inactivation of the Apc tumor suppressor [39, 40].

Many lines of evidence suggest that ITCs are highly promising for prevention of BC and BC recurrence. In this review, we consider the relationship of ITCs to cruciferous vegetables, selective delivery of ITCs to the bladder, cellular uptake of ITCs, preclinical evidence of cancer chemopreventive activities and molecular mechanisms of ITCs in BC cells and tissues, epidemiologic evidence of chemopreventive activities of ITC-rich cruciferous vegetables in the bladder, and the potential adverse effects of ITCs.

### ITCs and Cruciferous Vegetables

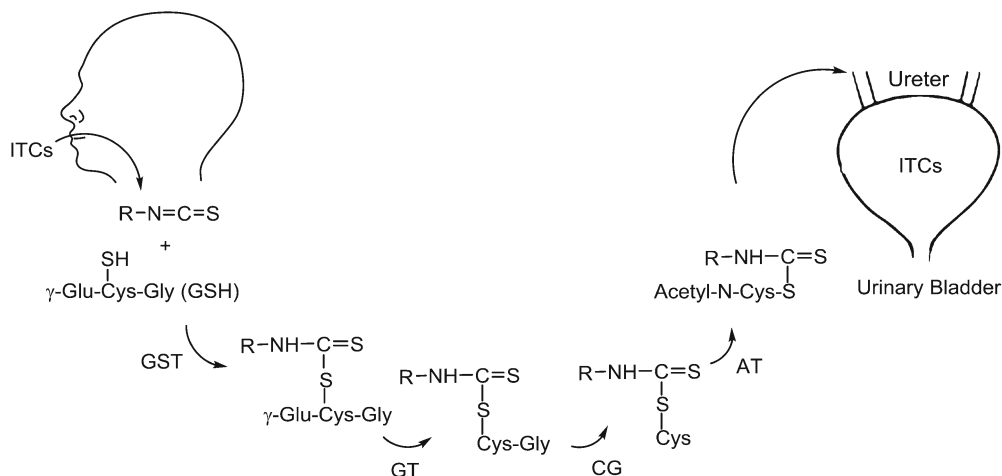
ITCs are characterized by the presence of an electrophilic functional group  $-N=C=S$  and a side chain unique to each ITC (Table 1). The chemopreventive actions of ITCs are mediated primarily by direct reaction of the carbon atom of the  $-N=C=S$  group with cellular nucleophiles, including many cancer-related proteins [41, 42] (also see section “Molecular Mechanisms by Which Cruciferous Vegetables and ITCs Target BC Cells”). The side chains of ITCs may modulate their chemopreventive activity by influencing the electrophilicity of the  $-N=C=S$  group and the lipophilicity of the ITC [41, 43].

Many commonly consumed cruciferous vegetables, such as broccoli, cabbage, garden cress, horseradish, mustard seed, wasabi, and watercress are rich sources of ITCs with significant cancer preventive activities (Table 1). ITCs, stored as glucosinolates ( $\beta$ -thioglucoside *N*-hydroxysulfates) in cruciferous vegetables, are generated through a myrosinase-catalyzed hydrolysis of their precursors when plant cells are damaged, first forming thiohydroximate-*O*-sulfonates (Table 1) [44]. Myrosinase, a thioglucoside glucohydrolase, is normally segregated from glucosinolates in plant cells [44]. While ITCs are products of spontaneous rearrangement of the thiohydroximate-*O*-sulfonates, nitriles, epithionitriles, and thiocyanates may form at the expense of ITCs due to the interaction of the intermediates with specifier proteins which exist in certain vegetables [45, 46]. Mild heating of vegetables (60–70 °C) inactivates the specifier proteins while preserving myrosinase, which increases the yield of SF in broccoli by 3–7-fold [47]. Cooking cruciferous vegetables in high heat destroys their myrosinase, and ITCs are also heat labile [48]. However, enteric microflora of both humans and animals possess myrosinase activity and may convert a significant portion of ingested glucosinolates to ITCs [16, 49]. Glucosinolate levels vary greatly among cruciferous vegetables. For example, total glucosinolate

contents in garden cress and Pe-tsai Chinese cabbage are 389 and 20 mg per 100 g fresh weight [50]. The average level of total ITC content ranges from 1.5  $\mu$ mol per 100 g fresh cauliflower to 61.3  $\mu$ mol per 100 g mustard greens [51].

### Metabolism and Urinary Disposition

ITCs, upon oral consumption, are primarily metabolized by the mercapturic acid pathway. They first conjugate with glutathione (GSH) via the cysteine sulfhydryl group, which occurs spontaneously but is further enhanced by glutathione *S*-transferase (GST). ITCs are substrates of several isoforms of GSTs, with GSTP1-1 and GSTM1-1 as the most efficient catalysts of ITCs and GSTM4-4 as the least effective [52]. The conjugates undergo multistep enzymatic modifications on the GSH moiety to form cysteinylglycine-, cysteine-, and *N*-acetylcysteine (NAC) conjugates; these are excreted in the urine (Fig. 1a) [53–61]. The organ sites for ITC metabolism have not been fully investigated, but tissue homogenates of both the liver and kidney catalyze the metabolic conversion [54]. The 1,2-benzenedithiol (BDT)-based cyclocondensation assay [62] was used to measure the absorption and bioavailability of ITCs as well as plasma and urine concentrations of ITC equivalents (ITC plus its metabolites formed in the mercapturic acid pathway) in humans and rodents in many of the studies described below. Plasma ITC levels increased very rapidly after a single ingestion of broccoli sprout extracts (BSE) containing 200  $\mu$ mol of ITCs (mainly SF) per person; concentration of ITC equivalents reached a maximum of 2.3  $\mu$ mol/L at 1 h and declined rapidly thereafter (half-life of 1.8 h), whereas the 8 h urinary concentration of ITC equivalent was 117  $\mu$ M [63]. Approximately 70 % of a SF dose was recovered as SF equivalents in 12 h of urine in human volunteers given BSE orally [64]. Likewise, approximately 70 % of a SF dose was recovered in the urine as SF equivalent within 12 h following oral administration of SF or SF-containing BSE to F344 rats, and the urinary concentrations of SF equivalents were 2–4 orders of magnitude higher than the corresponding plasma concentrations, respectively [65, 66, 67]. Bladder tissue uptake of SF was also significantly higher than that of other tissues in F344 rats fed with SF [65]. About 72 % of the dose was recovered in 24 h urine as NAC conjugates (60 % as NAC conjugate of SF and 12 % as NAC conjugate of ECN) after administration of SF at 50 mg/kg by intraperitoneal injection to male Sprague Dawley rats [56]. Approximately 95 % of the dose was recovered in 24 h urine as NAC conjugates (67 % as NAC conjugate of SF and 28 % as NAC conjugate of ECN) after administration of ECN by intraperitoneal injection to male Sprague-Dawley rats [56]. These results not only show extremely high bioavailability of SF and ECN but also show interconversion of SF and ECN *in vivo*. NAC conjugate was also the primary metabolite

**a** Orally consumed ITCs**b** Modulation of cellular targets by ITCs

|                                    |   |
|------------------------------------|---|
| ↑ Caspase-3&-9 [AITC, BITC, PEITC] | ↓ Cyclin A, B [AITC, BITC, PEITC, SF]                         |
| ↑ p-Bcl-2 (S-70) [AITC, BITC, SF]  | ↓ Cdc25C [BITC, SF]   |
| ↑ p27 [SF]                         | ↓ $\alpha$ -tubulin, $\beta$ -tubulin [AITC, BITC, PEITC, SF] |
| ↑ JNK [AITC, BITC]                 | ↓ Survivin [ECN, SF]  |
| ↑ Cyclin B1-Cdk1 complex [SF]      | ↓ Cox-2, PGE2 [SF]  |
|                                    | ↓ VEGF [AITC]   |
|                                    | ↓ EGFR [ECN, SF]  |

**Fig. 1** Selective delivery of ITCs to the bladder on oral consumption and modulation of cellular targets by ITCs in BC cells and tissues. **a** ITCs after oral administration are rapidly metabolized via mercapturic acid pathway, and the metabolites which are carriers of ITCs are excreted and concentrated in the urine. *GST* glutathione-S-transferase, *GT*  $\gamma$ -

overnight urines from human volunteers consuming 400  $\mu$ mol of glucoraphanin (the glucosinolate of SF) [58]. At least 42–54 % of the dose was recovered in the urine as NAC-AITC within 10–12 h of oral administration of 45–90  $\mu$ mol of AITC via horseradish or mustard paste in humans [61, 68]. In Fischer 344 rats, up to 80 % of orally administered AITC was recovered in 24 h urine, and tissue levels of AITC in the bladder were approximately 10-fold higher than in other organs [60]. In the same study, in B6C3F<sub>1</sub> mice, the major metabolite of AITC was thiocyanate ion, suggesting hydrolysis of AITC [60]. When AITC was administered to F344 rats bearing both orthotopic bladder tumors and subcutaneous tumors derived from the same cell line (AY-27 BC cells), tissue levels of AITC equivalent were three orders of magnitude higher in the orthotopic tumors compared to those in subcutaneous tumors, and urinary concentrations of AITC equivalent were also 2–3 orders of magnitude higher than in the blood [32]. Similarly, 54 % of the BITC dose was recovered as NAC-BITC in 10–12 h urine in human volunteers consuming

glutamyltranspeptidase, *CG* cysteinylglycinase, *AT* *N*-acetyltransferase. The organ sites for ITC metabolism have not been fully investigated. **b** ITCs modulate many proteins that are important for cancer growth and progression

pure BITC (14.4 mg or 96.5  $\mu$ mol) or fresh garden cress (25–27 g) per person [55]. Also, 65 % of the dose was recovered as NAC conjugate in 12 h urine in the above study. NAC-ITCs and the other conjugates formed in the mercapturic acid pathway are biologically similar to their parent compounds, as they are carriers of ITCs [69]. The half-time of dissociation of NAC-ITC in an aqueous environment at pH 7.4 and 37 °C is approximately 1–3 h [70]. Storage of urine in the bladder likely promotes the dissociation of NAC-ITCs and other ITC metabolites to ITCs. Collectively, orally dosed ITCs are excreted and markedly concentrated in the urine. As the vast majority of BC arises in the urothelium, dietary ITCs are attractive for the prevention of BC and BC recurrence.

**Cellular Uptake of ITCs**

AITC, BITC, PEITC, and SF accumulated rapidly in cultured cells with the peak intracellular concentration reached within

0.5–3 h of treatment, which is 100- to 200-fold higher than the extracellular ITC concentration [42]. The ITCs apparently enter cells by diffusion and rapidly conjugate with intracellular thiols, mainly GSH [41]. GSH, abundant in animal and human cells (0.2–10 mM) [71], serves as the major driving force for intracellular accumulation of ITCs [72]. Initial uptake rates of these ITCs correlate well with the non-enzymatic second-order rate constants of their conjugation reactions with GSH [72], and cellular GST enhances ITC uptake by catalyzing ITC conjugation with GSH [72]. ITC accumulation in cells may also cause GSH depletion [42]. ITCs may also bind to proteins via reaction with their cysteine sulfhydryl groups or with an amino group [41, 73]. ITC binding to cellular proteins may be influenced by its lipophilicity, concentration, and exposure time [41, 42, 72]. ITCs may also modulate cancer-related proteins via induction of reactive oxygen species [74].

High intracellular accumulation of AITC, BITC, and SF is followed by a rapid export by membrane transporter pumps such as multidrug resistance-associated protein-1 (MRP-1) and P-glycoprotein-1 (Pgp-1) [75, 76]. Interestingly, BITC, PEITC, and other ITCs (10–100  $\mu$ M), but not AITC and SF, inhibit Pgp-1, MRP-1, and breast cancer resistance protein-mediated cellular export of several anticancer agents in cultured cells, which likely results from inhibition of the ATPase of the transporters [77, 78]. This suggests that certain ITCs may help mitigate drug resistance. However, whether export of ITCs is in any sense self-limiting is not known.

### Chemopreventive Activities of Cruciferous Vegetables and ITCs in Preclinical Studies

Our discussion will be focused on the chemopreventive activities of cruciferous vegetables and ITCs that are particularly relevant to the prevention of NMIBC recurrence and progression. Summarized in Table 2 are cell lines and animal models that were employed to evaluate the inhibitory effects of ITCs on BC.

### Cell Studies

ITCs, including AITC, BITC, PEITC, and SF, inhibit the survival and proliferation of a wide array of animal and human BC cell lines [16, 22, 79, 80]. The growth inhibitory activities of ITCs are not specific to a particular cancer cell line, but they appear to inhibit cancer cells more than normal cells. For example, AITC inhibited human UM-UC-3 BC cells and rat AY-27 BC cells with an  $IC_{50}$  of 2.7–3.3  $\mu$ M (72 h treatment), but it was more than 21 times less effective on normal human bladder epithelial cells [32]. AITC did not elicit significant cell cycle arrest and apoptosis in normal human bladder epithelial cells, even at concentrations that are highly effective against their malignant counterparts [32]. Both SF and ECN were more toxic to human BC cells, including RT4 cells (derived from low-grade non-muscle-invasive cancer), J82 cells, and UM-UC-3 cells (derived from high-grade muscle-invasive cancers) than to normal bladder urothelial cells [31]. Growth inhibitory activities of ITCs are associated with cell cycle arrest, induction of apoptosis, inhibition of angiogenesis, and invasion [81, 82–84, 85, 86]. PEITC and SF also inhibit inflammatory response, including downregulation of cyclooxygenase-2 (Cox-2) and reduction of prostaglandin E2 level [87, 88]. Toll-like receptor 4 (TLR4) is closely involved in inflammation, and SF (10–20  $\mu$ M) downregulated TLR4 signaling via thiol-dependent inhibition of its oligomerization in macrophages [89].

### Animal Studies

ITCs have been evaluated in several animal models of BC. In rats treated simultaneously with BITC (0.001–0.1 % in diet) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN; at 0.005 % in drinking water for 40 weeks), BITC inhibited BBN-induced bladder tumorigenesis in a dose-dependent manner [90]. Dietary BSE (rats consuming total ITCs, mainly SF, at 40–160  $\mu$ mol/kg/day) reduced BBN-induced BC incidence by up to 61 % and tumor multiplicity by up to 77 % as well as reducing tumor size and delaying tumor progression [67]. In

**Table 2** Inhibitory activities of ITCs against BC cells in vitro and in vivo

| ITCs, vegetables and other agents                               | BC cell lines/BC animal models  | Reference No.                                 |
|---|---|---|
| AITC, AITC-rich mustard seed powder, NAC-AITC, AITC & celecoxib | Cell lines: UM-UC-3, T24, AY-27<br>Rats: orthotopic AY-27 BC model, subcutaneous AY-27 BC model   | [69, 81–83]<br>[32, 84, 85, 91]               |
| BITC  | Cell lines: UM-UC-3, T24<br>Rats: orthotopic BBN-induced BC model   | [69, 82, 83]<br>[90]                          |
| PEITC   | Cell lines: UM-UC-3, T24  | [69, 82, 83]                                  |
| SF, SF-rich broccoli sprout extracts, ECN                       | Cell lines: UM-UC-3, T24, RT4, J82, UM-UC-3, normal human urothelial cells, 5637<br>Rats: orthotopic BBN-induced BC model<br>Mice: subcutaneous UM-UC-3 xenograft model | [69, 82, 83, 95, 96, 101]<br>[67]<br>[31, 86] |

rats bearing orthotopic BC, initiated by transurethral inoculation of AY-27 cells, AITC or NAC-AITC at 10  $\mu\text{mol/kg}$  daily by gavage for 3 weeks inhibited tumor growth by 30–40 % and significantly inhibited tumor muscle invasion, while higher AITC doses were not more effective [32, 91]. In the same animal model, AITC-rich mustard seed powder (MSP-1), given by gavage at the daily AITC dose of 9  $\mu\text{mol/kg}$  (71.5 mg powder per kilogram), showed antitumor efficacy similar to that of pure AITC, although, as with AITC, a higher MSP-1 dose did not lead to greater effect [84]. Moreover, AITC in combination with celecoxib, a selective Cox-2 inhibitor, led to increased tumor inhibition in the orthotopic BC model mentioned above, compared with each agent used alone [85]. The combination regime was also more effective than each single agent in inhibiting microvessel formation and stimulating microvessel maturation in tumor tissues [85].

SF and ECN as well as BSE were evaluated in a subcutaneous xenograft model in mice, initiated by inoculation of UM-UC-3 BC cells. Mice bearing the UM-UC-3 xenografts were treated with SF or ECN (295  $\mu\text{mol/kg}$ ) daily by gavage or 2–4 % BSE in the diet (providing approximately 171–297  $\mu\text{mol}$  SF or its precursor and 25–40  $\mu\text{mol}$  ECN or its precursor per kilogram daily) for 2 weeks; SF, ECN, and BSE significantly inhibited tumor growth by 33–58 %; this effect was associated with decreased Ki67 staining and accumulation of SF/ECN metabolites in the tumor tissues [31]. When mice bearing subcutaneous UM-UC-3 xenografts were treated with a SF extract of broccoli sprouts (68  $\mu\text{mol}$  SF per kilogram) by gavage twice daily for 5 weeks, tumor growth was inhibited by 63 %, which was associated with decreased angiogenesis, and infiltration of eosinophils, basophils, and lymphocytes in the tumors indicative of enhanced immune reaction [86].

Inhibition of BC cells and tumors by the ITCs and ITC-rich vegetable extracts described above is accompanied by modulation of many molecular targets in the cells and tumor tissues. These changes are described in detail in the following section.

### Molecular Mechanisms by Which Cruciferous Vegetables and ITCs Target BC Cells

ITCs modulate a number of proteins involved in the growth and progression of BC (Fig. 1). Many other proteins are modulated by ITCs in other tumor types [24, 92, 93]. In UM-UC-3 cells, AITC, BITC, PEITC, and SF (7.5–30  $\mu\text{M}$ ) downregulated cyclin A or cyclin B1, consistent with cell cycle arrest in S phase or G2/M phase [82]. MSP-1 and NAC-AITC showed activities similar to those of AITC [32, 84]. In RT4 cells, both BITC and NAC-BITC downregulated Cdc25C and caused arrest in G2/M and S phases [69]. SF-rich BSE also arrested UM-UC-3 cells in G2/M and S phases, which was accompanied by downregulation of Cdc25C and disruption of mitotic

spindles [94]. However, SF and ECN (5–20  $\mu\text{M}$ ) were shown to cause only G2/M phase arrest in UM-UC-3 cells and other human BC cell lines (RT4 and J82) [31]. In human T24 BC cells, SF caused G0/G1 arrest, which was associated with upregulation of cyclin-dependent kinase (cdk) inhibitor p27 [95]. Moreover, in human 5637 BC cells, SF caused only M phase arrest, which was associated with induction of cyclin B1 and phosphorylation of Cdk1 [96]. Thus, cell cycle arrest in specific phases and modulation of cell cycle regulators by an ITC may vary with experimental conditions and cell lines. Tubulins and microtubules are essential for mitosis. AITC (7.5–30  $\mu\text{M}$ ) bound to multiple cysteine residues of  $\alpha$ -tubulin (Cys127, Cys 347, and Cys 376) and  $\beta$ -tubulin (Cys12, Cys239, Cys303, and Cys354) stimulated their ubiquitination and degradation in UM-UC-3 cells, which was associated with strong mitotic arrest [81]. NAC-AITC (7.5–15  $\mu\text{M}$ ) also downregulated  $\alpha$ - and  $\beta$ -tubulins in AY-27 cells [91]. BITC, PEITC, and SF (10–20  $\mu\text{M}$ ) bound to cysteine residues of  $\alpha$ - and  $\beta$ -tubulins when tested in human lung cancer cells, including Cys347 of  $\alpha$ -tubulin and Cys303 of  $\beta$ -tubulin, which led to proteasomal degradation of the tubulins [97, 98]. BITC, PEITC, and SF also inhibited tubulin polymerization [98, 99], but AITC showed no such effect [81].

AITC (7.5–30  $\mu\text{M}$ ) stimulated phosphorylation of B cell lymphoma 2 (Bcl-2) at Ser-70 through activation of c-Jun N-terminal kinases (JNK) in UM-UC-3 cells, which led to induction of apoptosis [81]. AITC did not modulate other Bcl-2 family proteins, including Bcl-2 homologous antagonist killer (Bak), B cell lymphoma-extra large (Bcl-xl), Bcl-2-associated death promoter (Bad), Bcl-2-associated X protein (Bax), and BH3-interacting domain death agonist (Bid) in UM-UC-3 cells, but it disrupted the association of Bcl-2 with Bax and induced mitochondria-mediated apoptosis, including the loss of mitochondrial transmembrane potential, cytoplasmic accumulation of cytochrome C, activation of caspases 9/3, and cleavage of poly(ADP-ribose)polymerase (PARP) and lamin B1 [81]. MSP-1 and NAC-AITC also stimulated apoptosis in UM-UC-3 cells and AY-27 cells [32, 84, 91]. AITC-induced apoptosis depends on mitotic arrest, and Bcl-2 phosphorylation is the molecular link between mitotic arrest and apoptosis in AITC-treated cells [81]. Bcl-2 phosphorylation also increased in UM-UC-3 cells after treatment with BITC and PEITC (7.5  $\mu\text{M}$ ) [83]. BITC stimulated JNK-dependent Bcl-2 phosphorylation, when tested in human T cell leukemia Jurkat cells [100]. Mitochondria play a key role in ITC-induced apoptosis. Besides Bcl-2 phosphorylation mentioned above, the apoptosis-inducing potentials of AITC, BITC, PEITC, and SF in UM-UC-3 cells correlate well with their ability to modulate mitochondria; they induce mitochondria translocation of Bak, disrupt the association of Bcl-xl with Bak and Bax in mitochondrial membrane, and cause the collapse of mitochondrial transmembrane potential and release of cytochrome C [83]. AITC-induced apoptosis in UM-UC-3

cells can be blocked by either a Bcl-2 mutant that does not respond to AITC or a caspase 9 inhibitor [81•]. Reactive oxygen species have also been implicated in apoptosis induction by ITCs. In human T24 BC cells, induction of apoptosis by SF is linked to a reactive oxygen species-mediated mitochondrial apoptosis pathway [101].

Many other proteins involved in apoptosis are also modulated by ITCs. Cox-2, a pro-inflammatory oncogene that plays a pivotal role in BC development, is frequently overexpressed in BC and is associated with stage, grade, recurrence, and prognosis of BC [102]. SF (5–20  $\mu\text{M}$ ) downregulated Cox-2 in T24 cells via activation of p38 and inhibition of NF- $\kappa\text{B}$  binding to the Cox-2 promoter [88, 103]. AITC does not modulate Cox-2, but AITC in combination with celecoxib, a selective Cox-2 inhibitor, was more effective than each agent used alone for inhibition of orthotopic BC growth and muscle invasion in rats [85•]. This finding suggests that SF may complement AITC for BC inhibition. Vascular endothelial growth factor (VEGF) stimulates cancer angiogenesis, invasion, and metastasis. MSP-1 (13–26  $\mu\text{M}$  of AITC) and NAC-AITC (7.5–15  $\mu\text{M}$ ) caused significant downregulation of VEGF in both UM-UC-3 cells and AY-27 cells [84, 91•]. In RT4 cells and UM-UC-3 cells, SF and ECN (5–20  $\mu\text{M}$ ) also downregulated survivin and epidermal growth factor receptor (EGFR) [31].

In vivo, inhibition of orthotopic BC in rats by AITC (10  $\mu\text{mol/kg}$  daily), MSP-1 (providing AITC at 9  $\mu\text{mol/kg}$  daily), or NAC-AITC (10  $\mu\text{mol/kg}$  daily), as described in the animal studies section above, was associated with significant downregulation of cyclin B1,  $\alpha$ -/ $\beta$ -tubulins, and VEGF, and activation of caspase 3 in the tumor tissues [32, 84, 91•]. Significant inhibition of subcutaneous UM-UC-3 tumors in athymic mice by SF (68  $\mu\text{mol/kg}$  twice a day for 5 weeks) was associated with activation of caspase 3, cytochrome C release, and downregulation of survivin [86]. The SF-treated mice also had tumors with reduced number of blood vessels [86].

### Epidemiological Evidence of Chemopreventive Activities of Cruciferous Vegetables in the Bladder

Several epidemiological studies have shown that intake of cruciferous vegetables is inversely associated with BC risk. In a case-control study involving 275 BC patients and 825 controls, significant inverse association between BC risk and consumption of raw cruciferous vegetables, including broccoli, cabbage, and cauliflower, was observed [adjusted odds ratio (OR) for high ( $\geq 1$  serving/month) versus low ( $< 1$  serving/month) category is 0.64; 95 % confidence interval (CI), 0.42–0.97] [104]. In a prospective study involving 47,909 men over 10 years, cruciferous vegetable consumption was inversely associated with BC risk [relative risk (RR)=0.49; 95 % CI, 0.32–0.75] [105]. A meta-analysis of five cohorts and five case-control studies also showed significantly

decreased risk of BC with increased intake of cruciferous vegetables [RR=0.80; 95 % CI, 0.69–0.92] [106•]. However, no study has directly examined the association of cruciferous vegetable intake and BC recurrence. In a retrospective cohort of 239 BC patients, broccoli intake, in particular raw broccoli, at least one serving per month, was significantly associated with reduced overall mortality [hazard ratio (HR), 0.57; 95 % CI, 0.39–0.83] and BC-specific mortality (HR, 0.43; 95 % CI, 0.25–0.74) [107]. These epidemiological findings are consistent with results from cell and animal studies discussed before and provide additional evidence that ITC intake may slow BC growth, progression, and recurrence.

### Potential Toxicity of ITCs

Several studies have shown adverse effects of certain ITCs in animals. F344 rats (male and female) consuming a diet containing 0.1 % of BITC, PEITC, or an analog for 1–14 days showed acute toxicity in the bladder, such as hemorrhage, inflammation, and necrosis, followed by gradual increase in the thickness of the bladder epithelium, and papillary or nodular hyperplasia [108]. Male F344 rats treated with BITC at 0.1 % in the diet for 40 weeks alone showed epithelial hyperplasia, although BITC strongly prevented BBN from inducing dysplasia, papilloma, or carcinoma [90]. F344/DuCrj rats (male and female) upon drinking water containing AITC or an AITC-rich horseradish extract (estimated AITC intake 30–40 mg/kg/day) for 13 weeks developed simple hyperplasia (100 %) and papillary or nodular hyperplasia (20–40 %) [109]. In another study, administration of AITC by gavage (12–25 mg/kg or 121–252  $\mu\text{mol/kg}$ ) daily 5 days per week for 103 weeks induced transitional cell papillomas (prevalence 4–8 %) in male F344 rats but not female rats, which was suspected to be due to higher urinary concentrations of ITC metabolites in the male rats [110]. In contrast, AITC given to F344 rats orally at 10  $\mu\text{mol/kg}$  daily inhibited orthotopic BC, while higher AITC doses were not more effective [32]. PEITC induced bladder toxicities only when its dietary level exceeded 0.01–0.05 % (approximately 37–183  $\mu\text{mol/kg}$  daily) for 32 weeks [111]. Given that orally consumed ITCs are concentrated in the urine and ITCs are highly electrophilic, the bladder damage discussed above may be mainly due to overdosing. It is important to note that the toxicities observed in animal studies result from doses that are several orders of magnitude greater than those observed in human epidemiologic studies [112].

### Concluding Remarks

Accumulating evidence, from both epidemiologic and preclinical studies, suggests that naturally occurring ITCs as well as

ITC-containing cruciferous vegetables and vegetable extracts may be useful for prevention of BC development and recurrence in humans. ITCs can be administered orally but are selectively delivered to the bladder via urinary excretion, contrasting with currently available intravesical agents that are delivered via a urethral catheter. Further studies are needed, in order to better understand the chemopreventive activity of ITCs and their mechanisms of actions, to identify biomarkers potentially useful for clinical evaluation of ITCs in patients, and to evaluate the chemopreventive efficacy of mechanism-based combination of multiple ITCs or ITC with other agents. Clearer understanding of the potential adverse effects of these compounds in humans is also needed. Additional population studies may further clarify the relationship between consumption of cruciferous vegetables and BC risk, risk of BC recurrence, and survival of BC patients. Rodent models may understate the cancer chemopreventive activities of ITCs in the bladder, as rodents are prone to frequent urination, whereas urine storage in the bladder in humans may facilitate the dissociation of ITC metabolites and increase the exposure of BC cells to ITCs in the urine. For this reason, the available pre-clinical and epidemiologic data suggest a need for clinical evaluation of ITCs or ITC-containing vegetable extracts in BC patients.

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#### Compliance with Ethics Guidelines

**Conflict of Interest** Omkara L. Veeranki, Arup Bhattacharya, Li Tang, James R. Marshall, and Yuesheng Zhang declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any new studies with human or animal subjects performed by any of the authors.

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