RESEARCH ARTICLE

Inhibition of bladder cancer by broccoli isothiocyanates sulforaphane and erucin: Characterization, metabolism, and interconversion

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Scope: Epidemiologic evidence suggests diets rich in cruciferous vegetables, particularly broccoli, are associated with lower bladder cancer risk. Our objectives are to investigate these observations and determine the role of isothiocyanates in primary or secondary bladder cancer prevention.

Methods and results: We initially investigate the mechanisms whereby broccoli and broccoli sprout extracts and pure isothiocyanates inhibit normal, noninvasive (RT4), and invasive (J82, UMUC3) human urothelial cell viability. Sulforaphane ($IC_{50} = 5.66 \pm 1.2$ μM) and erucin ($IC_{50} = 8.79 \pm 1.3$ μM) are found to be the most potent inhibitors and normal cells are least sensitive. This observation is associated with downregulation of survivin, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2/neu), G_2/M cell cycle accumulation, and apoptosis. In a murine UMUC3 xenograft model, we fed semipurified diets containing 4% broccoli sprouts, or 2% broccoli sprout isothiocyanate extract; or gavaged pure sulforaphane or erucin (each at 295 μmol/kg, similar to dietary exposure); and report tumor weight reduction of 42% ($p = 0.02$), 42% ($p = 0.04$), 33% ($p = 0.04$), and 58% ($p < 0.0001$), respectively. Sulforaphane and erucin metabolites are present in mouse plasma (micromolar range) and tumor tissue, with *N*-acetylcysteine conjugates as the most abundant. Interconversion of sulforaphane and erucin metabolites was observed.

Conclusion: This work supports development of fully characterized, novel food products containing broccoli components for phase I/II human studies targeting bladder cancer prevention.

Keywords:
Bladder cancer / Broccoli / Erucin / Isothiocyanates / Sulforaphane

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Abbreviations: Allyl ITC, allyl isothiocyanate; **ANOVA**, analysis of variance; **BBN**, *N*-butyl-*N*-(4 hydroxybutyl) nitrosamine; **BPE**, bovine pituitary extract; **CYS**, cysteine; **CYSGLY**, cysteinyl glycine; **ECN**, erucin; **EGFR**, epidermal growth factor receptor; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **GSH**, glutathione; **H&E**, hematoxylin & eosin; **HBCC**, human bladder cancer cell line; **hEGF**, human epidermal growth factor; **HER2/neu**, human epidermal growth factor receptor 2; **NAC**, *N*-acetyl-L-cysteine; **NHU**, normal human bladder urothelial cells; **PARP**, poly adenosine diphosphate ribose polymerase; **SFN**, sulforaphane; **SRB**, sulforhodamine B assay; **UPLC**, Ultra performance liquid chromatography

1 Introduction

Cancer of the urinary bladder is the fifth most common neoplasm in the United States [1] and presents a significant health care burden as the most expensive cancer to treat and monitor per patient, with an annual cost of $3.7 billion [2]. Tobacco smoking is the most important risk factor of bladder cancer and is a global concern due to expansion in tobacco use [3, 4]. The urothelium is the transitional cell epithelial lining of the bladder and is the origin of 90% of bladder cancers in the United States [5]. These transitional cell carcinomas arise and progress along two distinctive pathways representing two different clinical diseases: a "low-grade non-invasive" disease encompassing 80% of urothelial carcinomas and a "high-grade invasive" disease. The noninvasive disease is usually successfully treated with surgical resection, but has a 70% recurrence rate and a 10–20% chance of progression into a high-grade invasive disease. The invasive disease has a more

dismal prognosis with approximately 50% risk of death regardless of treatment [5, 6].

Bladder cancer exhibits several attributes making it an ideal target for prevention. These include its high recurrence rate, feasibility of accessing and monitoring the uroepithelium for tumors by cystoscopy, availability of predictive and diagnostic urine biomarkers [7], and the opportunity of delivering dietary components or pharmacologic chemopreventive agents through the urine, in addition to bloodstream, which may enhance anticancer activity.

Epidemiological studies support the potential role of dietary factors in bladder cancer risk, such as increased fluid intake and consumption of cruciferous vegetables, particularly broccoli [8–12]. In a large prospective cohort study, a 39% reduction in bladder cancer risk with ≥ 2 servings of broccoli/week was observed, when compared with <1 serving ($p = 0.009$) [9]. Glucosinolates are the parent phytochemicals in broccoli and are converted to isothiocyanates by myrosinase, an enzyme that is released when the plant's cell structure has been disrupted through chewing, chopping, or digestion [13]. Evidence suggests that isothiocyanates are a major group of active phytochemicals in broccoli and may possess cancer inhibitory properties [14–17].

In spite of strong epidemiologic data, there are limited in vitro and in vivo studies examining the potential inhibitory effects of cruciferous vegetables and their components on specific stages of bladder carcinogenesis. Broccoli sprout extract fed at 160 μmol/kg/day for 36 weeks of study resulted in a significant decrease in incidence, multiplicity, size, and progression of BBN-induced bladder cancer (where BBN is *N*-butyl-*N*-(4 hydroxybutyl) nitrosamine) [18]. This was mainly attributed to the induction of phase II enzymes and the detoxification of the chemical carcinogen. Similarly, sulforaphane (SFN) can inhibit 4-aminobiphenyl-induced DNA damage in RT4 bladder cancer cells and in mouse bladder tissue [19]. These studies, although very important, focus on broccoli isothiocyanates' ability to inhibit bladder cancer by detoxifying carcinogens and preventing DNA damage. However, it is possible that broccoli isothiocyanates may also impact bladder carcinogenesis by other mechanisms, particularly during the postinitiation phase [15]. Recently, it has been shown that allyl isothiocyanate (Allyl ITC)-rich mustard seed powder can inhibit bladder cancer growth and block muscle invasion by 34.5% in an orthotopic rat model [20]. A transplantable xenograft human bladder cancer model, looking at the potential of broccoli and its isothiocyanates to inhibit bladder tumorigenesis, has not been performed. This model especially applies to the secondary prevention approach in bladder cancer, where bladder cancer is inhibited in the postinitiation phase to prevent recurrence and progression [21]. In addition, comparison of a dietary approach (broccoli sprouts and broccoli sprouts extract) with pure isothiocyanates (SFN and erucin (ECN)) in bladder cancer prevention and/or treatment has not been elucidated.

There is also a strong need for information on in vivo isothiocyanate bioavailability, distribution to target organs and tumor tissue, as well as metabolism, along with defining plasma concentrations associated with bioactivity [22]. Furthermore, studies have predominantly focused on free SFN or SFN-GSH, with minimal studies focusing on other important isothiocyanates, such as ECN, and isothiocyanate metabolites produced through the mercapturic acid pathway [23, 24]. Herein, we test the efficacy of broccoli isothiocyanates both in vitro and in vivo, and assess their metabolism and tumor penetration in a xenograft murine model. We show that broccoli, broccoli sprouts, or their pure isothiocyanates inhibit the viability of specific bladder cancer cell lines, and this effect is at least in part through induction of apoptosis, modulation of cell cycle, and downregulation of antiapoptotic protein, survivin, and members of ErbB family receptor kinases, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2/neu). Next, we demonstrate that broccoli isothiocyanates, whether administered through diet or pure gavage, lead to significant inhibition of bladder tumorigenesis in a xenograft murine model, and that ECN demonstrates greater potency. In addition, we find that broccoli isothiocyanates are readily absorbed and document that metabolites are present in mouse blood and tumor tissues. We also report the interconversion between SFN and ECN metabolites in vivo. Our results further support the use of broccoli sprouts and/or their pure isothiocyanates in the prevention of bladder cancer and warrant further investigation in this regard.

2 Materials and methods

2.1 Pure isothiocyanates, broccoli, and broccoli sprout isothiocyanate extracts

2.1.1 Pure phytochemicals

R-, S-, and R,S-SFN, ECN (LKT Laboratories, Inc., St. Paul, MN, USA), Allyl ITC (Sigma-Aldrich, St. Louis, MO, USA), and iberin (MP Biomedicals, Santa Ana, CA, USA) were analyzed by Ultra performance liquid chromatography (UPLC)-MS/MS for purity and quality. Treatments were prepared as stock solutions in DMSO and diluted in culture medium. The same DMSO concentration (0.1%) was used in all treatments.

2.1.2 Glucosinolate and isothiocyanate extracts for cell culture

Glucosinolates were extracted from organic broccoli and broccoli sprouts (BroccoSprouts®) (Whole Foods, Columbus, OH, USA) [25] and quantified by LC-MS/MS, utilizing previously reported methods [26]. Isothiocyanate extracts for both broccoli and broccoli sprouts were obtained from glucosinolate extracts shaken with excess myrosinase (Sigma-Aldrich) in 95% 33 mM sodium phosphate buffer (pH 6.5–7.0, containing 2 g/L ascorbic acid and 0.01 M

magnesium chloride) and 5% tetrahydrofuran (THF) for 3 h and quantified by LC-MS/MS. Dilutions were made in THF and media, with a final THF concentration consistently at 0.125%.

2.1.3 Broccoli sprout diet preparations for animal studies

Two diets were produced for the murine study. (1) Broccoli sprout isothiocyanate extract diet: Broccoli sprouts were boiled in water for 30 min; sprouts were then filtered out and extract was cooled. Exogenous myrosinase was added and mixture was incubated for 3.5 h at 37C. The mixture was flash frozen at −40C and then lyophilized and milled into a fine powder. Total iberin, SFN, and ECN were quantified by LC-MS/MS. (2) Broccoli sprout diet: Broccoli sprouts were lyophilized and then ground into a fine powder. Glucoiberin, glucoraphanin, and glucoerucin concentrations were quantified by LC-MS/MS [26]. Diets were stored at −20°C and food was changed every other day for the duration of the study.

2.2 Cell culture

All human bladder cancer cell lines (HBCCs), RT4, J82, and UMUC3, were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. Normal human bladder urothelial cells (NHU) were purchased from Cambrex Bioscience Inc. (Walkersville, MD, USA) and cultured in serum-free, keratinocyte growth medium (KGM-2 SingleQuots), supplemented with bovine pituitary extract (BPE), human epidermal growth factor (hEGF), insulin, hydrocortisone, epinephrine, transferrin, and gentamicin/amphoterecin-B. All cells were grown as monolayer cultures at 37°C in a 95% air/5% CO_2 humidified atmosphere.

2.3 Cell viability

Cells were seeded in quadruplicate in 96-well plates in appropriate media, at optimized cell densities, for 1 week for NHU and 24 h for HBCCs, before treatment. After treatment with different concentrations of phytochemicals for 48 (HBCCs) or 72 h (NHU), cell viability was measured by sulforhodamine B assay (SRB) assay (Sulforhodamine B-Based In Vitro Toxicology Assay Kit, Sigma) per manufacturer's protocol, and percent cell viability was calculated. Optimal seeding density and the duration of treatment were determined by growth kinetics experiments, for NHU and each HBCCs, to accomplish a comparable treatment effect (data not shown).

2.4 Caspase 3/7 assay and cell cycle analysis

For caspase 3/7 assay, cells were handled in 96-well plates as above, and treated with SFN or ECN at 0, 5, and 10 μM concentrations for 48 h. Caspase-3/7 activity was assessed by the Apo-ONE Homogenous Caspase-3/7 Assay (Promega) per manufacturer's protocol. For cell cycle analysis, cells were seeded overnight in culture dishes, then treated with SFN or ECN at 0, 5, 10, and 20 μM concentrations for 48 h. The cells were harvested, washed, fixed in ethanol, stained with propidium iodide, and analyzed by flow cytometry using FACSCalibur and ModFit, as previously described [27].

2.5 Western blot analysis

Lysates of HBCCs treated with phytochemicals, in the same way as cell cycle experiments, were prepared for immunoblotting of poly adenosine diphosphate ribose polymerase (PARP), survivin, EGFR, HER2/neu, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Western blot analysis was performed as previously reported [28].

2.6 In vivo study

Mouse xenograft studies were carried out with strict adherence to protocols approved by the Institutional Animal Care and Use Committee of The Ohio State University (Animal Protocol 2007A0167-R1; Principal Investigator: A. Mortazavi). Female athymic nude mice ($FOXN1^{nu}$ 4–5 weeks of age) were obtained from Harlan laboratories and acclimated on AIN93G pelleted diet for 1 week. Mice were then subcutaneously inoculated with 0.05×10^6 UMUC3, in 0.1 mL of Matrigel (BD Biosciences; 50% [v/v] in serum-free medium), in the left and right dorsal flanks. Based on our previous experience with this model and the aggressive nature of this tumor, 3 days after injection, mice were randomized into one of these treatment groups (12 mice/group): (A1) Vehicle control soybean oil, oral gavage once daily; (A2) 295 μmol/kg body weight (bw) SFN (in soybean oil), oral gavage once daily; (A3) 295 μmol/kg bw ECN (in soybean oil), oral gavage once daily; (B1) Control AIN93G diet, fed ad libitum; (B2) AIN93G diet + 4% freeze-dried broccoli sprout, fed ad libitum; (B3) AIN93G diet + 2% freeze-dried broccoli sprout isothiocyanate extract, fed ad libitum. Mice were lightly anesthetized with isofluorane prior to daily gavage treatments. Mouse weights and tumor sizes were measured biweekly with tumor volume determined by using calipers and volumes calculated using the standard formula: $\text{width}^2 \times \text{length} \times 0.52$. Mice were sacrificed at 2 weeks, when tumors reached approximately 1.2 cm in diameter. At sacrifice, blood was collected and plasma isolated. Tumors were removed, measured, and weighed, and bladders were also collected, with half of the tissue flash frozen in liquid nitrogen and the other half fixed overnight in 10% neutral buffered

formalin and then transferred to 70% ethanol for histological use. Tumor and plasma were preserved for metabolite quantification studies in 0.2% formic acid. Two mice from each group were submitted to The Ohio State University Mouse Phenotyping Shared Resource for evaluation of gross and histologic pathology.

2.7 Histology

Formalin-fixed paraffin embedded tumor tissue from control and 4% broccoli sprout treated mice were sectioned and stained with hematoxylin & eosin (H&E). Ki67 staining was also performed per manufacturer's protocol with Ki67 primary antibody (1:50, rat monoclonal antibody, Dako Cytomation), biotinylated secondary antibodies, and Vector ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) used. The percentage of positive nuclei was then calculated based on the following formula: labeling index (%) = L / (L + C) × 100, where L = labeled cells and C = counterstained, unlabeled cells.

2.8 Metabolite quantification

Mercapturic acid pathway metabolites of SFN and ECN in plasma and tumor tissue were quantified by UPLC-MS/MS. GSH-SFN, CYS-SFN, and NAC-SFN (where CYS is cysteine and NAC is *N*-acetyl-L-cysteine) were purchased from Toronto Research Chemicals, Toronto, Ontario, Canada. GSH-ECN, CYSGLY-SFN, CYSGLY-ECN, CYS-ECN, and NAC-ECN (where CYSGLY is cysteinyl glycine) were synthesized as previously described [29]. Plasma samples were prepared as previously described [30], and tumor tissue samples were prepared by suspension in 10% (v/v) trifluroacetic acid (TFA) (0°C) with probe sonication for 5 sec and incubated at room temperature for 5 min prior to centrifugation at 20 000 × *g* for 5 min. Supernatants were injected directly for UPLC-MS/MS quantization. UPLC Acquity BEH C18 (2.1 × 100 mm, 1.7 μm) was used with a mobile phase of 0.1% (v/v) formic acid in water versus 0.1% (v/v) formic acid in acetonitrile at 0.45 mL/min, 40°C. Five microliter injection MS HPLC eluent was interfaced without flow splitting to a triple quadrupole mass spectrometer (Quattro Ultima, Micromass, UK) via an electrospray probe operated in positive mode. Reproducible chromatography and MS response could not be achieved with free ECN and thus it was omitted from the analysis.

2.9 Statistics

Statistical significance was tested by two-tailed Student's *t*-test or analysis of variance (ANOVA), followed by the Student-Newman–Keuls multiple comparisons test, using Instat software (GraphPad). Significance was set at $p < 0.05$.

3 Results

3.1 Quantification of glucosinolates reveals significantly higher levels in broccoli sprouts than mature broccoli

We characterized individual, intact glucosinolates in broccoli and broccoli sprouts by our previously developed LC-MS/MS method [26]. Broccoli sprouts employed in our studies have 6.4-fold higher level of total combined glucosinolates, as compared to broccoli. Glucoraphanin (precursor to SFN) is the predominant glucosinolate found in both broccoli and broccoli sprouts. Aliphatic glucosinolates, glucoiberin (precursor to iberin), and glucoerucin (precursor to ECN), are the second and third highest glucosinolates in broccoli sprouts. Indole glucosinolates, glucobrassicin (precursor to indole-3-carbinol) and neoglucobrassicin, are the second and third predominant glucosinolates in broccoli (Table 1).

3.2 Broccoli and broccoli sprouts isothiocyanate extracts and pure isothiocyanates significantly inhibit viability of noninvasive and invasive human bladder cancer cells

Glucosinolate extracts, from either broccoli or broccoli sprouts, show no effect on the growth of HBCCs. However, isothiocyanate extracts, produced by adding exogenous myrosinase to glucosinolate extracts, cause a significant dose-dependent decrease in cell viability of both RT4 and J82 cell lines ($p < 0.01$) (Fig. 1A). We show broccoli isothiocyanate extract at 7.3 μM (1 g vegetable/L) causes 28 ($p = 0.003$) and 37% ($p < 0.0001$) inhibition of viability in RT4 and J82 cells, respectively. Mirroring our quantification data, (Table 1) broccoli sprout isothiocyanate extracts show stronger inhibition by vegetable weight due to their higher glucosinolate and isothiocyanate content than broccoli. In RT4 cells, 5 μM of total isothiocyanate equivalent from broccoli sprout extract (0.05 g freeze-dried broccoli sprout/L) causes 17% inhibition of cell viability ($p = 0.008$) and 100 μM (1 g freeze-dried broccoli sprout/L) causes 88% inhibition of cell viability ($p < 0.0001$). In J82 cells, 10% inhibition is observed at 1 μM of broccoli sprout total isothiocyanate equivalent (0.01 g vegetable/L) ($p = 0.04$) and 82% inhibition is observed at 100 μM (1 g vegetable/L) ($p < 0.0001$). Broccoli sprout isothiocyanate extract has an IC_{50} of 21.5 ± 1.4 μM in J82 and 22.5 ± 1.9 μM in RT4 cells (Fig. 1A). Next, we compare individual pure isothiocyanates, SFN, ECN, Allyl ITC, and iberin, found in broccoli and broccoli sprouts, for their effects on the viability of HBCCs. Our previous experiments have shown that the optimal bladder cancer cell inhibitory effects of these compounds are achieved at 48 h of treatment, the time point that we choose for our in vitro studies. We first compare the effects of R-SFN (naturally

Table 1. Characterization of glucosinolates in broccoli and broccoli sprout extracts, as quantified by HPLC-MS/MS. Values represent the mean of two samples

Glucosinolate	Corresponding ITC	Broccoli		Broccoli sprout	
		μmol/g dry	% of total	μmol/g dry	% of total
Glucoraphanin	Sulforaphane	8.53	64.4	44.5	52.6
Glucoiberin	Iberin	0.03	0.23	21.3	25.2
Singrin	Allyl ITC	0.00	0.00	0.51	0.60
Glucoerucin	Erucin	0.01	0.08	9.02	10.7
Glucobrassicin	Indole-3-carbinol	2.19	16.5	0.54	0.64
Progoitrin	n/a	0.00	0.00	3.08	3.64
Gluconapin	n/a	0.15	1.11	1.34	1.59
Glucoalysin	n/a	0.06	0.45	0.06	0.08
Neoglucobrassicin	n/a	1.9	14.3	1.96	2.31
4-methoxy glucobrassicin	n/a	0.41	3.08	1.54	1.82
Gluconapoleiferin	n/a	0.02	0.15	0.76	0.90
Total		13.3	100	84.6	100

found in broccoli), S-SFN, and R,S-SFN (synthetic, racemic mixture) on the cell viability of HBCCs and find no difference between isomers in their inhibitory effects of HBCCs ($p > 0.8$) (data not shown). Thus, we utilize synthetic R,S-SFN for all remaining experiments. Of all isothiocyanates found in broccoli and broccoli sprouts, SFN is the most potent inhibitor ($IC_{50} = 5.66 \pm 1.2$ μM in UMUC3 cells, 48 h) followed by ECN ($IC_{50} = 8.79 \pm 1.3$ μM in UMUC3 cells, 48 h), and then Allyl ITC and iberin. The extent of RT4 cell viability inhibition by 20 μM of isothiocyanates at 48 h is: SFN 64% ($p < 0.001$), ECN 59% ($p < 0.001$), Allyl ITC 28% ($p < 0.001$), and iberin 12% ($p < 0.001$). We see similar trends of inhibition in J82 and UMUC3 cell lines (Fig. 1B). Finally, we compare a panel of HBCCs with NHU to determine if isothiocyanates can selectively inhibit HBCCs over NHU. We find SFN to inhibit NHU least (62% inhibition by 20 μM, and IC_{50} not reached) followed by RT4 cells ($IC_{50} = 11.2 \pm 0.3$ μM), then J82 cells ($IC_{50} = 7.7 \pm 1.8$ μM), and finally UMUC3 cells ($IC_{50} = 5.66 \pm 1.2$ μM). NHU cells are also least sensitive to ECN ($IC_{50} = 19 \pm 1.7$ μM) with increased sensitivity by RT4 cells ($IC_{50} = 16 \pm 0.8$ μM, with 7% more inhibition, $p = 0.02$), followed by J82 cells ($IC_{50} = 11.3 \pm 0.1$ μM, with 12% more inhibition than NHU, $p = 0.042$), and finally UMUC3 cells ($IC_{50} = 8.79 \pm 1.3$ μM, with 23% more inhibition than NHU, $p = 0.005$) (Fig. 1C).

3.3 SFN and ECN induce cell cycle arrest and apoptosis in human bladder cancer cells

To elucidate potential contributing mechanisms of action by which broccoli isothiocyanates can inhibit bladder cancer cell viability, we studied the ability of these compounds to modulate the cell cycle and induce apoptosis. We first determined the effects of isothiocyanates on the cell cycle of HBCCs (48-h treatment). We observe a significant, dose-dependent accumulation of cells in the G_2/M phase. In RT4 cells, 20 μM SFN leads to a 2.6-fold increase of cells in the G_2/M phase compared to control ($p < 0.0001$) and 20 μM ECN leads to a 2.1-fold increase ($p = 0.0025$). In addition, J82 cells treated with 20 μM SFN undergo a threefold increase of cells in the G_2/M phase ($p = 0.0006$) and a 2.7-fold increase with 20 μM ECN treatment ($p < 0.0001$), with UMUC3 cells following similar trends. Although, we observed a slight trend toward accumulation of cells in the S-phase in some of the cell lines treated with SFN and/or ECN, this was not statistically significance ($p > 0.1$) (Fig. 2). Treatment of HBCCs with SFN and ECN also results in a significant dose-dependent increase in caspase 3/7 activity (Fig. 3A) and the cleavage of their important target, PARP (Fig. 3B and C), indicating the activation of apoptotic cascades. In RT4 cells, 10 μM SFN treatment for 48 h leads to a 2.5-fold increase in caspase 3/7 activity ($p = 0.003$) and 10 μM ECN leads to a 3.2-fold increase ($p < 0.0001$), as compared to control (Fig. 3A). Mirroring these findings, PARP cleavage increases 2.3-fold in RT4 cells treated with 20 μM SFN ($p = 0.013$) and twofold when treated with 20 μM ECN ($p = 0.002$). In UMUC3 cells, PARP cleavage increases 3.6-fold with 20 μM SFN treatment ($p < 0.001$) and 20 μM ECN causes a fourfold increase ($p = 0.003$) (Fig. 3B and C). In addition, we studied survivin expression, an important antiapoptotic protein and a predictor of bladder cancer disease progression [31]. We observe a significant dose-dependent decrease in survivin expression, by SFN and ECN, in all HBCCs. In RT4 cells, 20 μM SFN leads to a 51% decrease in survivin expression ($p < 0.0001$) and 20 μM SFN leads to a 44% decrease ($p < 0.0001$). In addition, in UMUC3 cells, survivin expression is downregulated by 35% with 20 μM SFN treatment ($p < 0.0001$) and 69% with 20 μM ECN treatment ($p = 0.0003$) (Fig. 3B).

Tyrosine kinase receptors, EGFR and HER2/neu, are shown to be overexpressed in urothelial tumors and their degree of expression is also associated with cancer progression

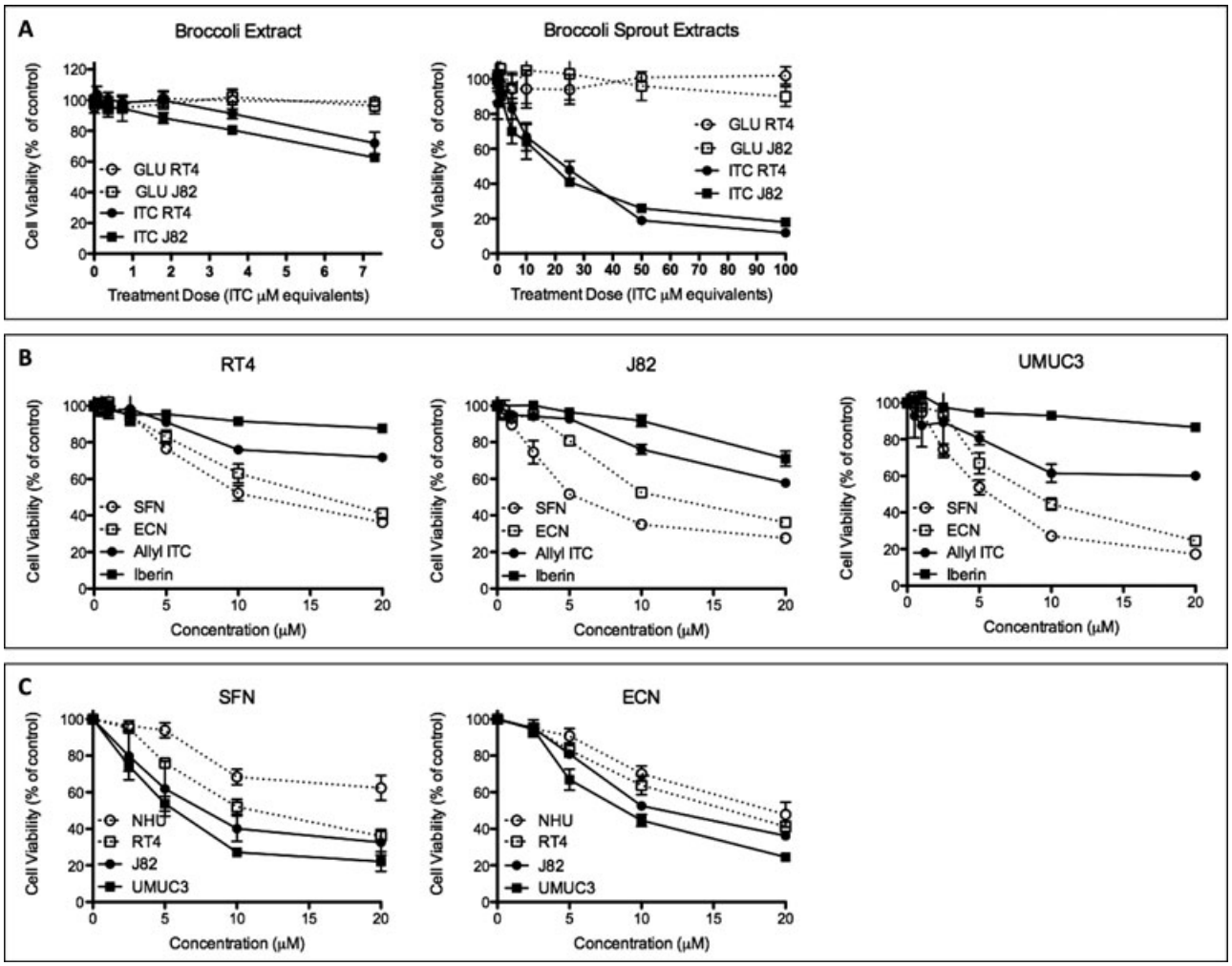
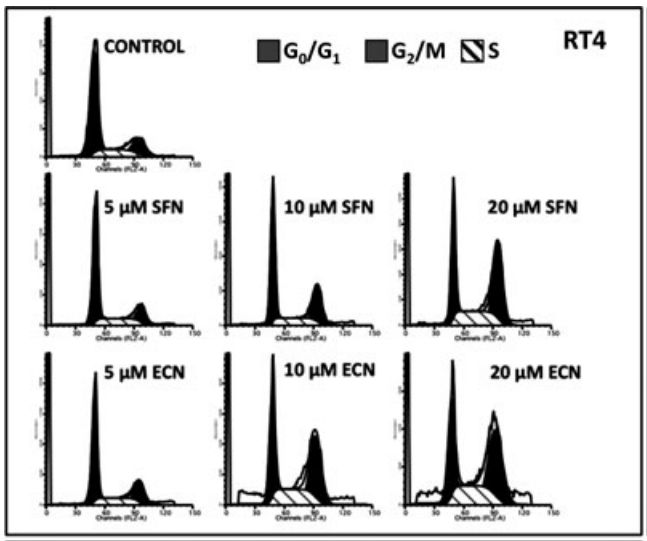
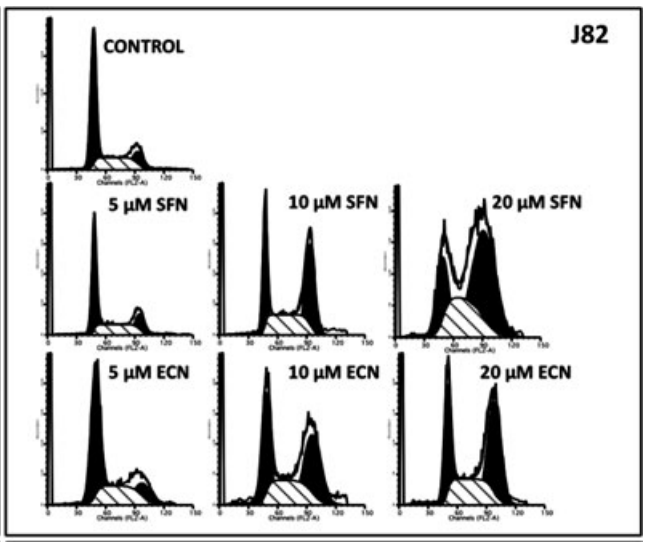
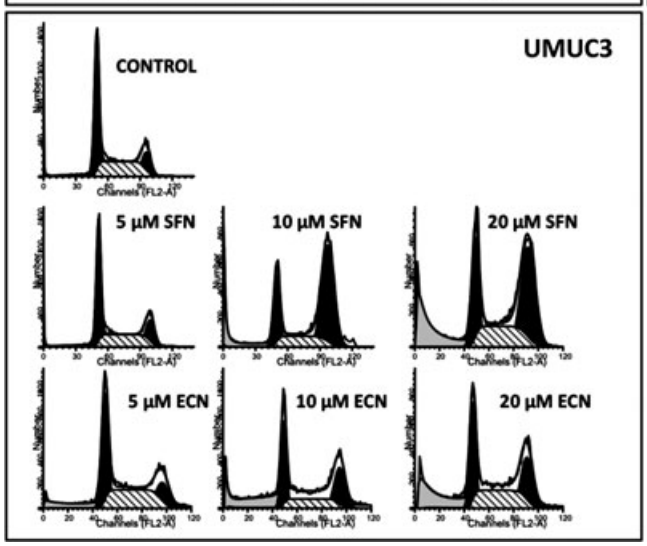


Figure 1. Broccoli and broccoli sprout isothiocyanates significantly inhibit viability of human bladder cancer cell lines (HBCCs). Growth inhibitory effects of broccoli and broccoli sprout glucosinolates and enzymatically hydrolyzed glucosinolates (isothiocyanates) and pure isothiocyanates are assessed by sulforhodamine B (SRB) assay. (A) The effects of glucosinolate and isothiocyanate extracts from broccoli and broccoli sprouts on the cell viability of RT4 (noninvasive) and J82 (invasive) HBCCs. Serial dilutions of broccoli and broccoli glucosinolate and isothiocyanate extracts are prepared and total glucosinolate and isothiocyanate equivalents calculated based on HPLC-MS/MS quantification (1 g freeze-dried broccoli/L = 14.3 μM glucosinolate equivalent and 7.28 μM isothiocyanate equivalent; 1 g freeze-dried broccoli sprouts/L = 100 μM glucosinolate equivalent and 100 μM isothiocyanate equivalent). Broccoli treatments include 0.00728, 0.0728, 0.364, 0.728, 1.82, 3.64, and 7.28 μM isothiocyanate equivalents and broccoli sprout treatments include 0.1, 1, 5, 10, 25, 50, and 100 μM isothiocyanate equivalents. Treatments are performed for 48 h. (B) The most abundant isothiocyanates found in broccoli and broccoli sprout isothiocyanate extracts, sulforaphane (SFN), erucin (ECN), allyl isothiocyanate (Allyl ITC), and iberin, are compared for antiproliferative effects in RT4 (noninvasive), and J82 and UMUC3 (invasive) HBCCs. Doses of 0, 0.5, 1, 2.5, 5, 10, 20 μM are used for 48 h of treatment. (C) Comparative inhibition of cell viability by SFN and ECN in normal human urothelial/bladder cells (NHU) and a panel of HBCCs ranging from noninvasive low-grade (RT4) to invasive high-grade (J82 and UMUC3). NHU are treated for 72 h and HBCCs treated for 48 h, to adjust for the difference in the growth curves of these cells. Points = means; bars = ±SD, ($n = 3$).

and poor prognosis [32]. Therefore, we study the ability of broccoli isothiocyanates to modulate the expression of these receptors, which may show new mechanisms of action for these compounds and may have clinical implications. Interestingly, we find that both SFN and ECN significantly downregulate EGFR and HER2/neu expression in the invasive UMUC3 cell line, but not to an appreciable level in noninvasive cell line RT4. In RT4 cells, 20 μM of SFN ($p = 0.11$) or ECN ($p = 0.25$) does not lead to significant changes in EGFR expression. However, in UMUC3 cells, EGFR protein expression is downregulated 54% by 20 μM SFN ($p = 0.01$) and 73% by 20 μM ECN ($p = 0.0002$). Furthermore, in RT4 cells, 20 μM SFN causes a 10% decrease of HER2/neu expression ($p = 0.013$) and no significant changes with 20 μM ECN ($p = 0.33$). In contrast, HER2/neu is downregulated 90% in UMUC3 cells by 20 μM SFN ($p < 0.0001$) and 83% by 20 μM ECN ($p = 0.001$) (Fig. 3B and C). Similar trends were observed in J82 cells (data not shown).







	G_0/G_1 (%)		S (%)		G_2/M (%)	
RT4						
Control	61	1	20	1	18	1
5 μM SFN	56	5	23	1	18	1
10 μM SFN	51	2	21	1	28	2 *
20 μM SFN	27	4	26	1	47	5 *
5 μM ECN	56	2	23	1	21	2
10 μM ECN	33	3	27	1	43	4 *
20 μM ECN	25	4	27	1	38	8 *
J82						
Control	55	1	31	1	15	1
5 μM SFN	50	1	34	1	15	1
10 μM SFN	27	1	35	1	40	1 *
20 μM SFN	22	2	40	1	45	9 *
5 μM ECN	51	1	34	1	16	1
10 μM ECN	31	2	35	1	36	2 *
20 μM ECN	29	3	32	1	41	5 *
UMUC3						
Control	45	2	39	1	14	1
5 μM SFN	43	1	39	1	18	1
10 μM SFN	24	4	22	2	55	5 *
20 μM SFN	27	1	26	2	43	3 *
5 μM ECN	40	1	40	2	17	1
10 μM ECN	33	2	39	1	33	1 *
20 μM ECN	32	2	37	2	30	2 *

Figure 2. Sulforaphane (SFN) and erucin (ECN) treatment leads to accumulation of human bladder cancer cell lines in the G_2/M phase of the cell cycle. RT4 (noninvasive low-grade) and J82 and UMUC3 (invasive high-grade) human bladder cancer cells are treated with vehicle control (DMSO) or 5, 10, and 20 μM of SFN or ECN for 48 h and then stained with propidium iodide for cell cycle analysis through flow cytometry. A significant dose-dependent accumulation of cells in the G_2/M phase is observed. Each histogram is representative of three independent experiments, and each was done in duplicate samples. ($n = 3$), $^*p < 0.05$.

Overall, SFN and ECN exhibit similar impacts on apoptosis and cell cycle progression ($p > 0.1$).

3.4 Broccoli sprout isothiocyanates significantly inhibit bladder cancer in vivo

Observations of the inhibitory effects of broccoli isothiocyanates in vitro, led us to examine the relevance of our work in an in vivo xenograft model. We conducted two separate in vivo experiments to elucidate the effects of two distinct phytochemical delivery approaches, meaning a pure isothiocyanate gavage versus a dietary administration of whole broccoli sprouts. In the gavage arm, we compared pure isothiocyanates of SFN or ECN to the vehicle control; and in the dietary arm, we compared the whole broccoli sprouts or broccoli sprouts isothiocyanate extract to the control diet. We chose to use a dose of 295 μmol SFN/kg body weight based on previous xenograft studies in other cancer models showing safety and efficacy at this dose [33, 34]. Encouraged by our in vitro data, we chose to compare ECN's potency at an equivalent dose to SFN.

We incorporated broccoli sprout isothiocyanate extract into a semipurified diet to provide a similar overall exposure as in our gavage of pure isothiocyanates. Our HPLC-MS/MS quantification shows that our broccoli sprout isothiocyanate extract contained 197 μmol isothiocyanate/g extract with 63% SFN, 28% iberin, and 8.4% ECN. Given that female

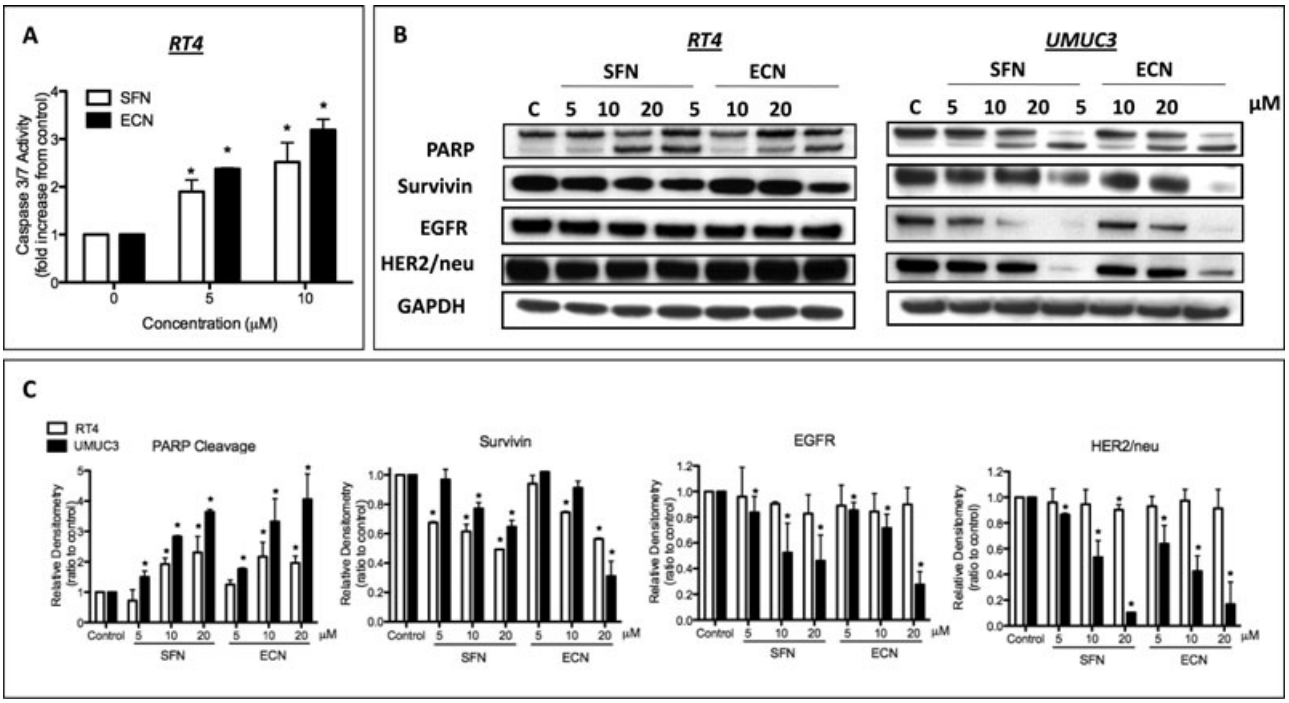


Figure 3. Induction of apoptosis and modulation of ErbB family of receptors by sulforaphane (SFN) and erucin (ECN) in human bladder cancer cell lines. Noninvasive (RT4) and invasive (UMUC3) bladder cancer cells are treated with vehicle control (DMSO) or 5, 10, and 20 μM of SFN or ECN for 48 h. (A) Dose-dependent induction of apoptosis by SFN and ECN, is evident by caspase 3/7 activity. Points = means; bars = ±SD, ($n = 3$), *$p < 0.05$. (B) Western blot analysis of dose-dependent effects of SFN and ECN on PARP cleavage, and expression of survivin, EGFR and HER2/neu. Each immunoblot is representative of three independent experiments. (C) Relative expression levels of cleaved PARP, survivin, EGFR, and HER2/neu. Amounts of immunoblotted proteins were quantified by densitometry and normalized against that of GAPDH. Points = means; bars = ±SD, ($n = 3$), *$p < 0.05$.

athymic nude mice eat approximately 3 g diet/day and weigh on average 25 g, we mixed 2% freeze-dried broccoli sprout isothiocyanate extract into AIN93G diet so that mice would consume approximately 11.82 μmol total isothiocyanates/day or 473 μmol total isothiocyanates/kg body weight/day (297 μmol/kg bw SFN, 132 μmol/kg bw iberin, and 39.7 μmol/kg bw ECN). We chose 2% to match our gavaged treatments of SFN and ECN with total SFN consumed.

A second dietary intervention provided whole freeze-dried broccoli sprouts. Our quantification of whole freeze-dried broccoli sprouts used in our animal study revealed 51 μmol total glucosinolate/g sprout with 68.5% glucoraphanin, 21% glucoiberin, and 10.3% glucoerucin. This batch differed in phytochemical content of that quantified in Table 1, which points to the variability of glucosinolates between batches of sprouts and highlights the importance of quantification when doing experiments of this nature. We mixed 4% by weight of broccoli sprout into AIN93G diet so mice would consume approximately 6.12 μmol total glucosinolates/day or 245 μmol total glucosinolates/kg body weight/day (171 μmol/kg bw glucoraphanin, 51 μmol/kg bw glucoiberin and 25.21 μmol/kg bw glucoerucin). In this case, we matched total glucosinolates to the SFN and ECN gavaged treatment concentrations we administered, as we did not want to administer an unreasonably high percentage of broccoli sprouts (4% over 8%) in mouse diet, for hopes of better translation into future human clinical trials.

By 2 weeks of treatment, we see a significant inhibition of UMUC3 tumor weight in all treated groups, with the greatest inhibition seen in the ECN-treated group. We see a 42% decrease in final tumor weight with 2% broccoli sprout isothiocyanate extract treatment ($p = 0.02$), and a 42% decrease with 4% broccoli sprout treatment ($p = 0.04$), as compared to the control diet (Fig. 4A). Also, we see a 33% decrease with 295 μmol SFN/kg/day treatment ($p = 0.04$), and a 58% decrease with 295 μmol ECN/kg/day treatment ($p < 0.0001$), as compared to the vehicle control soy bean oil (Fig. 4A). Tumor growth rates follow similar trends as final tumor weights; with 46% inhibition by 2% isothiocyanate extract treatment ($p = 0.03$), 39% inhibition by 4% sprout treatment ($p = 0.15$), 42% inhibition by SFN treatment ($p = 0.36$), and 82% inhibition by ECN treatment ($p = 0.11$) (Fig. 4A). To assess at least the minimum of proliferation inhibition in vivo, we chose the least inhibited treatment group (4% broccoli sprout) to perform Ki67 staining, a marker of cell proliferation, on harvested tumors and observe a 50% decrease in Ki67-positive cells ($p < 0.0001$) (Fig. 4B). There is no overt evidence of toxicity in serum chemistry or cell blood counts and no

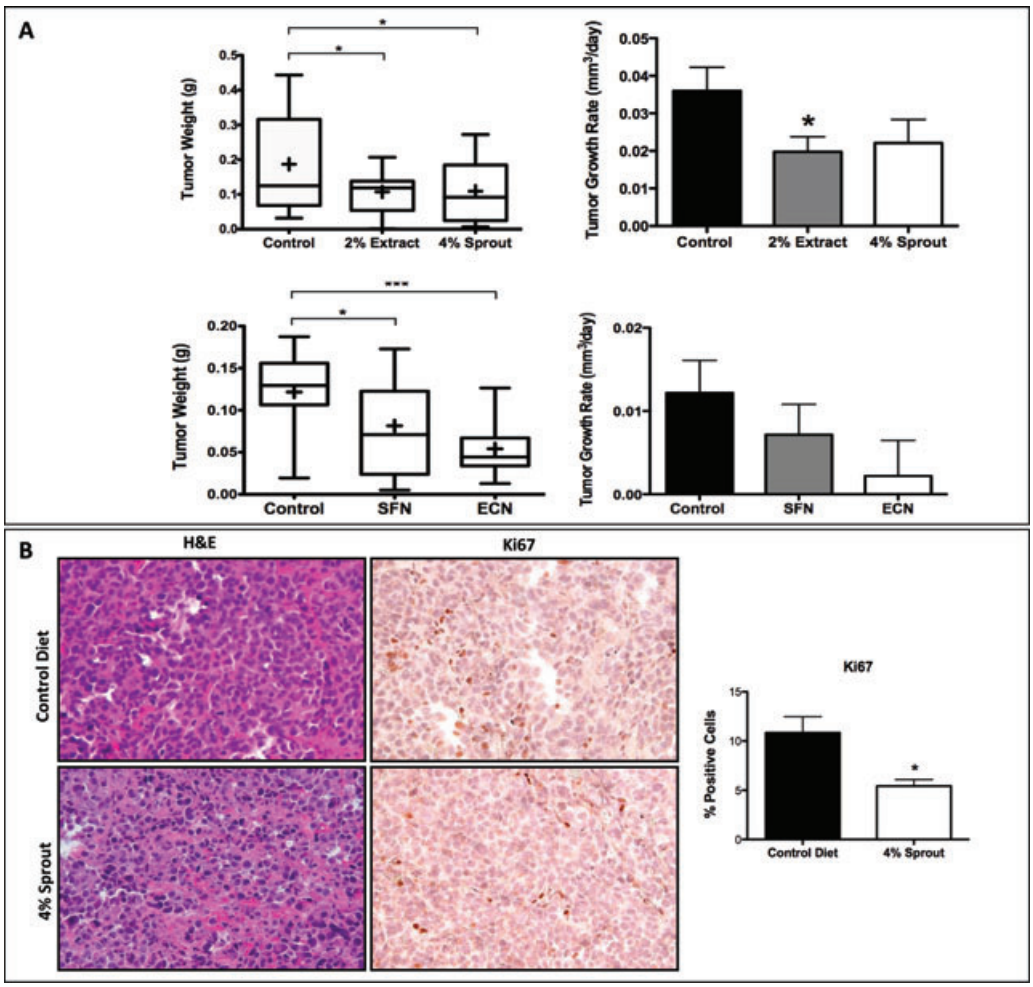


Figure 4. Broccoli sprouts and their isothiocyanates cause inhibition of bladder cancer in vivo. Female athymic nude mice ($n = 12$/group) were injected with UMUC3 (invasive) human bladder cancer cells in each flank and later randomized to one of these treatment groups: (A1) Vehicle control soybean oil, oral gavage once daily; (A2) 295 μmol/kg body weight sulforaphane (SFN) (in soybean oil), oral gavage once daily; (A3) 295 μmol/kg body weight erucin (ECN) (in soybean oil), oral gavage once daily; or (B1) control AIN93G diet, fed ad libitum; (B2) AIN93G diet + 2% freeze-dried broccoli sprout isothiocyanate extract, fed ad libitum; (B3) AIN 93 G diet + 4% freeze-dried broccoli sprout, fed ad libitum; for 2 weeks. (A) At sacrifice, tumors are harvested and weighed. Box plots represent final tumor weights, with plus signs indicating means. Tumor growth rates (mm^3/day) are also quantified during the course of treatment. Error bars for tumor weight represent standard deviation and for growth rate represent standard error (for means, $*p < 0.05$, $***p < 0.001$). (B) Tumors from control diet and 4% broccoli sprout diet-treated mice are stained with hematoxylin & eosin (H&E) showing tumor morphology or Ki67 (cell proliferation marker) with background hematoxylin stain, images are at 400× magnification. Proliferative index is the percentage of Ki67-stained cells. Data represent six independent samples, with three image fields counted per sample and error bars represent standard deviation ($*p < 0.05$, $n = 6$).

significant change in mouse weight between groups throughout the study ($p > 0.4$).

3.5 Isothiocyanates are efficiently absorbed and metabolized, with detectable metabolites in blood and bladder tumor tissue; and SFN and ECN are readily interconverted in vivo

Due to our observation that broccoli isothiocyanates treatment has the ability to inhibit bladder cancer growth in vivo, we become interested in assessing the bioavailability and metabolism of these compounds in our animal model. It is important to understand absorption and metabolism of these compounds in order to properly translate this work into future human clinical trials. We utilize UPLC-MS/MS to quantify SFN and ECN metabolites in mouse plasma and tumor tissues. Metabolites of SFN and ECN are absent in both control groups and present in plasma and tumors of all treated groups, with dietary and pure phytochemical treatments resulting in micromolar plasma metabolite concentrations with no significant differences between these groups

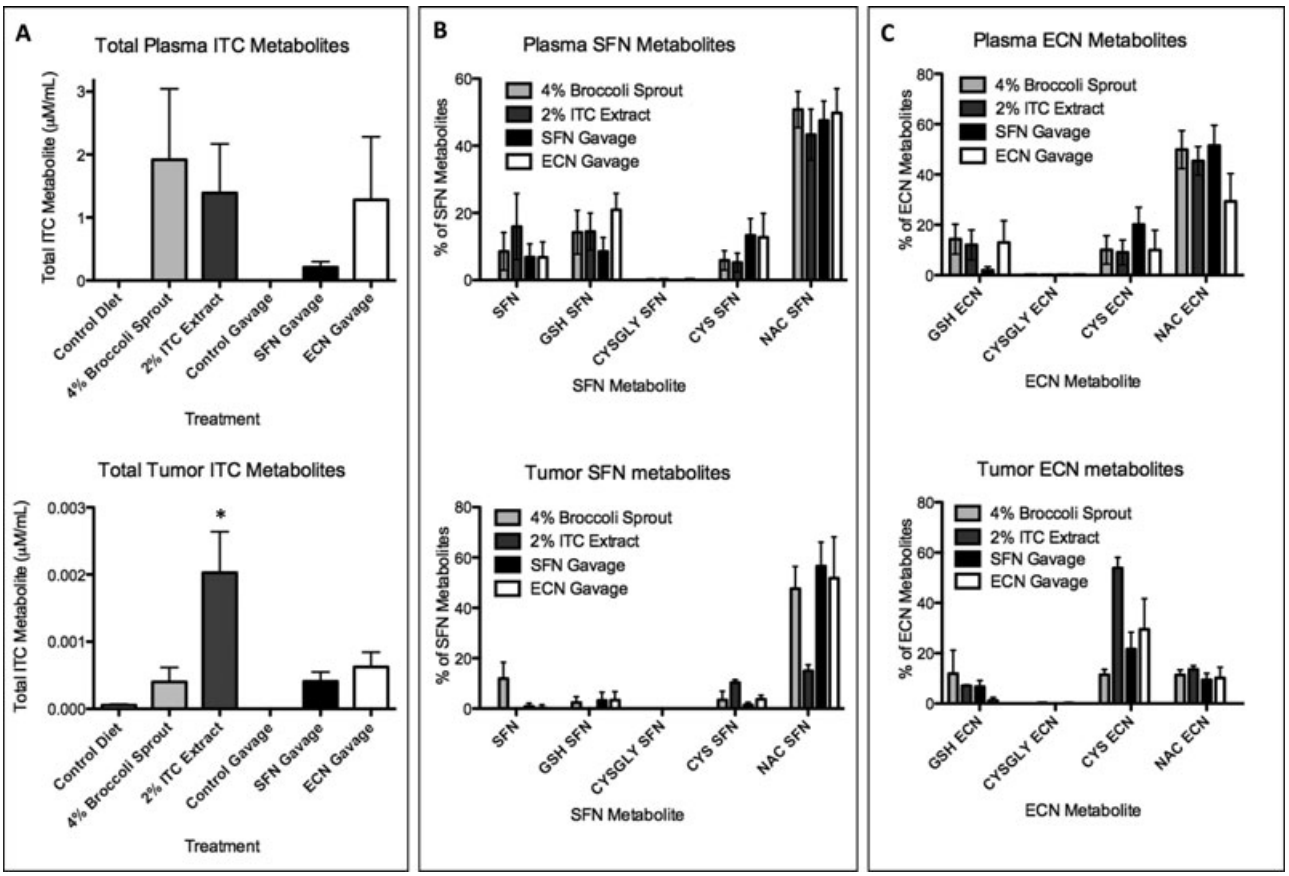


Figure 5. Broccoli isothiocyanates are efficiently absorbed, metabolized, and penetrated tumors and sulforaphane (SFN) and erucin (ECN) are readily interconverted. Total and individual isothiocyanate metabolites are quantified by UPLC-MS/MS in plasma and UMUC3 tumors from our xenograft study. (A) The sum of all SFN and ECN metabolites is represented in each group (μM/mL supernatant). (B) Analysis of SFN metabolites through the mercapturic acid pathway in dietary and gavaged treatment groups in plasma and tumor tissue. Evidence of interconversion of ECN into SFN metabolites is apparent in gavaged treatment groups. (C) Analysis of ECN metabolites through the mercapturic acid pathway in gavaged treatment groups in plasma and tumor tissue also reveals robust interconversion of SFN into ECN. Data represent at least four mice with error bars representing standard error (*$p < 0.05$) GSH, glutathione; CYSGLY, cysteinyl glycine; CYS, cysteine; NAC, *N*-acetylcysteine conjugates.

($p > 0.2$). There is also significant accumulation of broccoli isothiocyanate metabolites in tumor (Fig. 5A) and bladder tissues (data not shown) at picomolar range, with greatest absorption with the broccoli sprout isothiocyanate extract group over pure phytochemical treatment ($p < 0.05$). *N*-acetylcysteine conjugates of SFN and ECN are the most abundant metabolites in all ($p < 0.0001$), except for the tumors in ECN-gavage group, where CYS-ECN is the most abundant. Following *N*-acetylcysteine metabolites, CYS and gluathione conjugates are present, as well as small amounts of detectable cysteinylglycine conjugates and unconjugated SFN. Interestingly, we observe a robust interconversion of SFN and ECN, as both metabolites were present in the plasma and tumors of mice gavaged pure individual compounds (Fig. 5B and C).

4 Discussion

There remains a strong need for the improvement of bladder cancer prevention and treatment modalities. Our future objective is to develop a novel food product, rich in cruciferous vegetable bioactives, that can be employed in phase I/II clinical trials in those at risk of bladder cancer or recurrence following cystoscopic removal. In order to move forward, we conducted critical preclinical studies to define relevant analytic and tissue biomarkers. Our studies demonstrate the antitumor effects of broccoli, broccoli sprouts, and their pure isothiocyanates, using in vitro and in vivo models of bladder cancer. Most critically, we define the concentration of metabolites of these compounds in plasma and tumor tissue that are associated with anticancer bioactivity. A study

comparing the antibladder cancer effects of dietary administration of whole broccoli sprouts, broccoli sprout isothiocyanate extracts or pure isothiocyanates gavage has not previously been reported. Furthermore, there has been a strong focus in the literature on the ability of isothiocyanates to induce phase II enzymes, thereby aiding in the detoxification of carcinogens and thus leading to a cancer preventive response [17, 18, 35]. However, a study on the ability of broccoli isothiocyanates, particularly SFN and ECN, to inhibit the growth of established bladder cancer, outside the context of carcinogen detoxification, through the use of a human xenograft bladder cancer model has not been reported.

In order for in vitro and in vivo studies utilizing vegetable extracts to be meaningful, it is important to characterize phytochemicals present in these extracts, especially given the variation inherent in foods due to variations in genetics, growing conditions, and processing [36]. Herein, we first determine the amounts of individual glucosinolates present in broccoli and broccoli sprout extracts. The broccoli sprouts we employed show a greater than sixfold higher isothiocyanates concentrations than broccoli [25]. Thus, broccoli sprouts are an excellent choice for developing a food product for future human studies. Furthermore, our xenograft studies show that the use of 4% freeze-dried broccoli sprout diet (containing 51 μmol glucosinolate/g) leads to the same level of tumor inhibition as the 2% broccoli sprout isothiocyanate extract diet (containing 197 μmol isothiocyanate/g), with similar resulting levels of plasma isothiocyanates. Glucosinolates in the broccoli sprouts were likely converted into active isothiocyanates as a result of the release of endogenous myrosinase during the grinding process. This suggests there may be a level of maximal absorption of glucosinolates and isothiocyanates, and that a higher dose of administration may not lead to a greater beneficial effect. Furthermore, we find broccoli sprouts to have higher concentrations of glucoerucin, the precursor to ECN, than broccoli. Interestingly, our data show ECN to be the most potent inhibitor of bladder tumorigenesis in our xenograft model. This is a novel finding, because although ECN has been shown to induce phase II detoxification enzymes in the urinary bladder of rats [35], it has never been shown to inhibit bladder carcinogenesis.

Through our cell viability data, we show broccoli and broccoli sprout glucosinolates do not have an effect on bladder cancer cell viability, while isothiocyanates do, supporting present evidence for the importance of glucosinolate conversion to isothiocyanate for their anticancer activity [14, 15]. Furthermore, of the major isothiocyanates found in broccoli and broccoli sprouts, we show SFN and ECN are the most potent inhibitors of viability, causing significant induction of apoptosis and cell cycle arrest, which is also supported by previous work [35, 37, 38]. Our cell cycle analysis mirrors previous findings of arrest in the G_2/M and S phase through downregulation of Cdc25c and disruption of the mitotic spindle in bladder cancer cell lines treated with broccoli sprout isothiocyanate extract [39]. We also show that EGFR, HER2/neu, survivin and Ki67, important biological proteins, and prognostic markers for bladder cancer disease progression [31], are significantly inhibited by broccoli isothiocyanates.

We show that broccoli isothiocyanates, whether administered through diet as whole broccoli sprouts or broccoli sprout isothiocyanate extract or through gavage as pure isothiocyanates (SFN or ECN), can strongly inhibit aggressive, rapidly growing UMUC3 bladder xenograft tumor, without causing any apparent toxicity. Overall, it appears that consumption of phytochemicals, either through pure phytochemical gavage or dietary-based administration, inhibits the bladder tumorigenesis at a fairly similar rate. Through the use of our xenograft study, we also report that broccoli isothiocyanates have the ability to inhibit bladder carcinogenesis outside the context of carcinogen detoxification and also that isothiocyanates can inhibit bladder cancer, postinitiation. Future studies should employ the use of novel transgenic models of bladder cancer [40–42] to study the potential preventive benefits of these compounds.

We also show that isothiocyanates are detectable in mouse plasma and tumor tissue when administered through diet or by gavage, indicating effective absorption and tumor penetration. The bioavailability of ECN has not been studied to date [43]. We found that ECN gavage, broccoli sprout, and broccoli sprout isothiocyanate extract groups all had comparable plasma total isothiocyanate metabolite levels (1–2 μM) and comparable tumor inhibition. Most importantly, these effective plasma levels are achievable in humans [44, 45]. For reasons that are unclear, the SFN gavage group shows lower plasma concentrations ($\leq$1 μM) and less tumor inhibition, although not to a significant level. Tumor metabolite levels are comparable among the groups, except the isothiocyanate extract group, which had significantly higher concentrations.

Analysis of plasma and tumor tissue, collected 12–18 h after gavage of isothiocyanates, indicates sustained plasma and tumor levels, as supported by previous reports [18]. Interestingly, NAC-SFN is the predominant metabolite found in both plasma and tumor tissue and NAC-ECN was the predominant metabolite found in plasma while tumors had predominantly CYS-ECN. These thiol-conjugated isothiocyanates have been shown to dissociate into free isothiocyanates under physiologic conditions [46]. In addition, NAC-conjugated isothiocyanates are the predominant urinary metabolite of dietary isothiocyanates and have been shown to be equally potent at inhibiting human bladder cancer cell proliferation as free isothiocyanates [47]. Interestingly, the concentrations of broccoli isothiocyanate metabolites in tumor and bladder tissues are in the picomolar range, and yet biologically active, they are significantly less than the effective concentrations needed for in vitro experiments (micromolar range). This observation is very important for future animal and human studies. We also observe a robust interconversion of SFN and ECN, which has previously been reported in two rat studies and has been shown to occur in humans [48–50]. ECN was partially converted to SFN by oxidation reaction and SFN to ECN by reduction reaction. Both species are present when either is administered, suggesting equilibrium between these two

forms. We were unable to detect appreciable interconversion of these compounds in vitro (data not shown), supporting that this is largely an in vivo phenomenon. The cause and biological effects of the robust interconversion between SFN and ECN in vivo, which may occur by liver metabolism or conversion by gut microflora, deserve further study.

Overall, we report that broccoli and broccoli sprout isothiocyanates, especially SFN and ECN, can significantly inhibit bladder cancer growth both in vitro and in vivo. These compounds are readily bioavailable and their active metabolites can be detected in plasma and tumor tissues. This work supports continued preclinical research including prevention studies in murine transgenic models of human bladder cancer. This work also supports the development of novel food products, with a particular focus upon broccoli sprouts and the use of sophisticated processing techniques to enhance conversion of glucosinolates to active components. In addition, future clinical studies can include 'primary prevention', in high risk patients, such as heavy smokers; and 'secondary prevention', to decrease the rate of recurrence and progression of disease in established bladder cancer patients. Human investigations, should they show positive results, will significantly contribute to improving patients' outcomes, saving lives, and lowering health care costs.

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