

***In Vitro* and *In Vivo* Inhibition of Human Bladder Cancer by Broccoli Isothiocyanates: Characterization, Absorption, Metabolism and Interconversion**

Written by: Besma Abbaoui

The Integrated Biomedical Science Graduate Program, College of Medicine

Advisor: Dr. Steven K. Clinton

Division of Medical Oncology, College of Medicine

Introduction

Bladder cancer, the fourth most common cancer in men and the ninth in women, places a significant global healthcare burden with over 350,000 new cases diagnosed worldwide per year and approximately 145,000 deaths (1, 2). In the United States 2.35% of individuals born today will be diagnosed with bladder cancer in their lifetime (3, 4). In addition, bladder cancer has been estimated to be one of the most expensive cancers to treat in the USA with \$3.7 billion in direct costs (5). Tobacco smoking is the most important determined risk factor of bladder cancer, with 2- to 4-fold greater relative risk, and is a global concern due to expansion in tobacco use. Other risk factors include occupational and environmental carcinogen exposures (4, 6, 7).

The urothelium is the transitional epithelial lining of the bladder and is where 90% of bladder cancers, referred to as Transitional Cell Carcinoma (TCC), are derived (8, 9). There are two types of transitional cell carcinoma which are thought to progress through different mechanistic pathways: a non-invasive variant which encompasses approximately 80% of urothelial carcinomas and an invasive variant. The non-invasive variant, when treated with surgical resection and local immunotherapy, has a 5-year survival rate of almost 90%. However, this cancer has an approximately 70% chance of recurring and a 10-20% chance of developing into

invasive disease (10). The invasive form has a grimmer prognosis with greater than 50% of patient death from metastases within two years of diagnosis. Treatment is unsuccessful in 95% of patients and the 5-year survival rate is approximately 6% (9).

Dietary factors may play a significant role in the development of bladder cancer. A landmark series of epidemiological studies (11-14) included an examination of fruit and vegetable intake and the incidence of bladder cancer, reporting that intake of cruciferous vegetables, particularly broccoli, had a strong inverse association with bladder cancer risk (12). When compared to those consuming <1 serving of broccoli/week, consumption of 1 serving/week led to a decreased risk of 0.71 and ≥ 2 servings/week had a decreased risk of 0.61 ($p=.009$). The next necessary step is to test these findings from epidemiological studies in pre-clinical models and define mechanisms of action.

Broccoli (*Brassica oleracea italica*) is part of the cruciferous vegetable family and includes commonly consumed vegetables such as cauliflower, cabbage and brussel sprouts. Isothiocyanates are phytochemicals found in broccoli that have been shown to have anti-cancer effects. They include compounds such as sulforaphane, erucin, allyl isothiocyanate and iberin (15). In intact plants, isothiocyanates are stored as inert precursors called glucosinolates (16). Glucosinolates are converted to isothiocyanates when they are hydrolyzed by myrosinase, an enzyme that is released when the plant's cell structure has been disrupted through chewing, chopping or digestion (17). Young broccoli plants, especially sprouts, have 20-50 times the levels of preventative glucosinolates than more mature market-stage plants (18).

Collectively, recently published data suggests that broccoli and broccoli sprout isothiocyanates can be absorbed by the body, may reach μM concentrations in the blood, accumulate in tissues and be maintained to achieve an anti-cancer effect (19). Some evidence

also shows that active ITCs are capable of reaching the bladder epithelium via the urine (20). However, there is a need for more *in vivo* pharmacokinetic and metabolic studies before such work can be applied and translated into humans.

There is growing evidence that cruciferous vegetables and their phytochemicals, including isothiocyanates, have anti-cancer properties. *In vitro*, broccoli isothiocyanates have been shown to inhibit viability in a multitude of human cancer cells, inducing apoptosis and cell cycle arrest (21-23). There are far fewer *in vivo* studies, with predominant work in cancers such as prostate and colon both showing inhibition of tumor growth as a result of broccoli phytochemical treatment (24-27). The bladder cancer field possesses very limited *in vivo* studies with broccoli, however one rat study using a freeze-dried aqueous extract of broccoli sprouts showed a dose-dependent inhibition of bladder cancer development induced by the carcinogen BBN and maintenance of normal tissue (24). Scant clinical studies exist, however the few that do show a lack of toxicity of broccoli isothiocyanate administration in humans (28, 29). Clearly, there is a great need for additional *in vivo* pre-clinical studies in order to test the potential of these foods for the development of human clinical trials.

Most mechanistic studies of the potential preventative effects of broccoli and ITCs focus on their ability to induce phase II enzymes and inhibit Phase I enzymes, leading to the detoxification of carcinogens as well as the prevention of carcinogen activation, respectively (30, 31). However, *in vitro* and *in vivo* studies, where cancer is inhibited by these compounds without carcinogen induction, indicates that there must be other mechanisms of action at play. There is recent evidence, that ITCs may act as histone deacetylase inhibitors (26, 32-34) altering epigenetic regulation of gene expression over time. Overall, however, much research needs to be done in order to uncover how these powerful compounds may act as anti-bladder cancer agents.

The epidemiological association between high cruciferous vegetable intake and decreased risk of bladder cancer warrants further preclinical studies of the potential of these compounds to inhibit bladder cancer utilizing *in vitro* and *in vivo* models. The ability of broccoli compounds to inhibit non-carcinogen induced bladder cancer has not been well-studied.

Herein we report that broccoli isothiocyanates can inhibit human bladder cancer cell viability *in vitro* in a panel of bladder cancer cell lines ranging from superficial (RT4) to invasive (J82, UMUC3) as well as induce apoptosis and modulate the cell cycle, leading to cell accumulation in the G2/M phase. In addition, UMUC3 xenograft mice treated with either 4% freeze-dried broccoli sprouts, 2% freeze-dried broccoli sprouts extract; or gavaged daily with 295 $\mu\text{mol/kg}$ sulforaphane or 295 $\mu\text{mol/kg}$ erucin lead to a significant at least 40% reduction in tumor weight after 2 weeks of treatment when compared to the control groups and that this inhibition was associated with inhibition of cell proliferation (Ki67 staining) and induction of apoptosis (PARP cleavage). Finally, we show that broccoli isothiocyanates are readily absorbed and metabolized in mouse blood and reach micromolar concentrations and that there is an apparent interconversion between sulforaphane and erucin metabolites *in vivo*.

Materials and Methods

Reagents: Sinigrin, glucoiberin, progoitrin, glucoerucin, and glucotropaeolin were purchased from the Department of Chemistry, The Royal Veterinary and Agricultural University, Denmark. Thioglucosidase (myrosinase) extracted from white mustard seed and BDT were purchased from Sigma-Aldrich (St. Louis, MO). All solvents used were of HPLC grade and were purchased from Fisher Scientific (Fairlawn, NJ). Organic broccoli and BroccoSprouts[®] were purchased fresh from a local supermarket (Whole Foods, Columbus, Ohio). *N*-acetyl cysteine (NAC) was

purchased from Fluka (Switzerland). Sulforaphane (SFN), erucin, iberin, phenethyl ITC, and allyl ITC were purchased from LKT Laboratories, Inc. (St. Paul, Minnesota). Iberverin was purchased from Alfa Aesar (Ward Hill, MA). All solvents used were of HPLC grade and were purchased from Fisher Scientific (Fairlawn, NJ).

Extraction of glucosinolates for quantification: A modified previously reported procedure was used to extract the GLUs from the freeze-dried vegetables (35). Briefly, broccoli and broccoli sprouts were frozen at -80°C and lyophilized for 48h. The freeze-dried vegetables were milled into fine powders using a mortar and pestle and samples were extracted in 70% aqueous methanol and sonicated for 15 min at 70°C and centrifuged at $15,000g$ for 15 min, and the supernatant was removed. Supernatants were then concentrated under vacuum with a rotary evaporator (Büchi R110) with a maximum temperature of 50°C and then frozen at -80°C , and lyophilized until dry. Triplicate samples were extracted and analyzed, and data are represented as average \pm standard deviation.

LC-ESI/MS/MS of intact glucosinolates: All mass spectra were obtained using a Quattro Ultima ion-tunnel mass spectrometer (Micromass, Manchester, UK) coupled to a Waters 2696 HPLC system equipped with a 996 photo-diode array (PDA) detector (Waters Associates, Milford, MA). HPLC separation was performed on a 250×4.6 mm ($5 \mu\text{m}$) Luna C_{18} (2) reversed-phase column (Phenomenex Ltd., Torrance, CA) with a Phenomenex security guard column. A linear gradient mobile phase from 100% A (water containing 0.5% trifluoroacetic acid) to 15% B (acetonitrile) in 10 min, to 40% B in 5 min, to 50% B in 5 min, and returned to 100% A in 5 min was used to elute the analytes at a flow rate of 1 ml/min. Approximately $100 \mu\text{L}/\text{min}$ of the

HPLC eluant separated by a micro-splitter (Upchurch Scientific, Oak Harbor, WA) was delivered to the Z-spray ESI source. Negative ion tandem mass spectrometry (MS/MS) was conducted to detect GLUs with selected reaction monitoring (SRM). The mass spectrometer was tuned by direct infusion of standard sinigrin producing maximum abundant precursor ion m/z 358 ($[M-H]^-$) and fragment ion m/z 97 ($[SO_3H]^-$) signals (approximately in equivalent abundance) during MS/MS. The mass spectrometric conditions are as follows: capillary, 2.35 kV; cone voltage, -35 V (RF-1, 50 V); desolvation gas temperature, 450 °C at a flow of 16.5 L/min; source temperature, 120 °C; collision energy, 18 eV. The following transitions were used to assay ten individual GLUs: glucoiberin (422>97), sinigrin (358>97), progoitrin (388>97), glucoerucin (420>97), glucoraphanin (436>97), gluconapin (372>97), glucoalysin (450>97), glucobrassicin (447>97), neoglucobrassicin (477>97), 4-methoxy glucobrassicin (477>97). Glucotropaeolin (408>97), not found in cruciferous vegetables and previously used as an internal standard in a European Committee method (12), was used as the internal standard in the current study. A dwell time of 0.08 s was used for each transition. Standard calibration curves were established by triplicate analyses (seven concentration levels), and deviations from the mean calculated concentrations over three runs were within 15% from the nominal concentrations. At the lower limit of quantification (LLOQ) level, the deviation was within 20%. For GLUs in vegetables, glucoiberin, sinigrin, progoitrin, and glucoerucin were quantified using their corresponding standard calibration curves; because standards were not available for the remaining compounds, glucoraphanin and glucoalysin were estimated using the calibration curve of glucoiberin, based on the structural similarities of the three compounds. Similarly, gluconapin was estimated using the calibration curve of sinigrin. The three indole GLUs (glucobrassicin, neoglucobrassicin, and 4-methoxy glucobrassicin) were estimated using the calibration curve of

the internal standard (glucotropaeolin). To avoid any irreproducibility caused by conditions such as HPLC eluant splitting ratio, temperature, or the detector condition, all the analyses were conducted in sequence without disruption.

Extraction of glucosinolates and conversion to isothiocyanates: To ensure consistency among extracts, a large scale method was used for extractions. Twelve 1.5 g samples of freeze-dried broccoli or broccoli sprout powder were extracted in 50-ml screw-top centrifuge tubes using 25 ml 70% aqueous methanol and sonicated for 15 min at 70°C. After being cooled in an ice bath, the extract was centrifuged at 15,000g for 15 min, and the supernatant was removed using a syringe. The extraction procedure was completed three times and the supernatants of samples 1-6 and 7-12 were combined; samples 1-6 were intended for GLUs and samples 7-12 were intended for ITCs. 400 ml of each of the two extracts were concentrated under vacuum to ~120 ml with a rotary evaporator (Büchi R110) with a maximum temperature of 50°C. Both extracts were divided evenly among 36 11-ml glass screw-top vials (72 vials total), frozen at -80°C, and lyophilized until dry. Two GLU samples were analyzed by HPLC-MS-MS(36) and the remaining samples were stored at -20°C until further use. Immediately prior to analysis, 2 vials of ITC extract was resolubilized with 5 mL of 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, and hydrolyzed by adding excess myrosinase. The vial was sealed tightly and shaken for 3 hours at room temperature.

Synthesis of N-acetyl cysteine derivatives of isothiocyanates: Before quantifying the ITCs in the extract, some samples were first conjugated to NAC as a method of stabilization (Figure 3.1).

This derivative was prepared by combining the strategies of previously published methods(53, 54). 50 μ L of pure ITCs (\sim 1 mM) or freshly hydrolyzed ITC extracts from broccoli or broccoli sprouts were added to 1.9 ml 20 mM phosphate buffer (pH 8.5) containing approximately 5 times molar excess NAC in a glass screw-top vial. The vial was flushed with nitrogen, sealed, and allowed to react overnight at room temperature with shaking. To protonate the carboxyl group, the pH was lowered to 2.5 with 1M hydrochloric acid, drop wise. The conjugate was extracted 3 times with ethyl acetate, and the supernatants were pooled and dried under a stream of nitrogen gas in a 15-ml screw-top centrifuge tube. The resulting residue was stored at -20 °C until ready to use.

Total isothiocyanate quantification: The amount of total ITC was determined based on previously reported methods(54-56). The samples were quantified using a standard curve of 1,3-benzodithiole-2-thione (BTT). BTT was synthesized by combining \sim 1 mM propyl ITC in methanol and 10-fold molar excess BDT in phosphate buffer (pH 8.5), and reacting for 2 hours at 65°C. The resulting solution was cooled to room temperature before extracting 3 times with methylene dichloride, pooling the extracts. The methylene dichloride solution was saturated with sodium sulfate to remove any remaining water or water-soluble compounds. The solution was filtered through a 0.2 μ m nylon filter (Waters Associate, Milford, MA), dried under a stream of nitrogen gas, and stored at -20°C until ready to use. Immediately before subjecting to HPLC analysis (20 μ L injection), the BTT was resolubilized in 100 μ L methylene dichloride and 4500 μ L methanol, vortex mixed, and again filtered with a 0.2 μ m nylon filter. The reaction scheme is shown in Figure 1.2. 50 μ L of myrosinase-hydrolyzed broccoli or broccoli sprout extract, either free or conjugated with NAC (resolubilized in 50 μ L methanol), and 500 μ L of 100 mM

phosphate buffer (pH 8.5) were combined in an 11-ml glass screw-top vial. The vial was flushed with argon, and 100 μ L of 10 mM BDT in de-oxygenated methanol was added to the vial while continuously flushing with argon. The tube was sealed, vortex mixed, and shaken for 2 hours at 65°C. It was then cooled to room temperature, centrifuged, and injected into the HPLC (40 μ L). Analysis was performed on a Waters 2696 HPLC system equipped with a 996 photo-diode array (PDA) detector (Waters Associates, Milford, MA) using a 250 \times 4.6 mm (5 μ m) Luna C₁₈ (2) reversed-phase column (Phenomenex Ltd., Torrance, CA) with a Phenomenex security guard column with a flow rate of 1 ml/min. Injections were made into an 80% aqueous methanol mobile phase, and the eluants were monitored from 200 to 450 nm, quantifying at 365 nm.

Extraction of glucosinolates and conversion to isothiocyanates for cell culture: GLUs were extracted with 70% aqueous methanol from freeze-dried organic broccoli and broccoli sprouts. The GLU extracts were either stored at -20 °C or hydrolyzed to ITCs using myrosinase. The GLU extracts were quantified using LC-MS/MS (36), while the ITC extracts were quantified using the cyclocondensation assay (36). For ITC extracts used in the sulforhodamine B (SRB) assay, the GLUs were hydrolyzed with myrosinase and this solution directly applied to cells.

Cell Culture: RT4, J82 and UMUC3 human bladder cancer cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin. Normal human bladder urothelial cells were purchased from Cambrex Bioscience Inc. (Walkersville, MD) through special contractual order and cultured in serum free, keratinocyte growth medium (KGM-2 SingleQuots), supplemented with BPE, hEGF, insulin, hydrocortisone, epinephrine,

transferrin and gentamicin/ amphoterecin-B from Cambrex. All cells were grown as monolayer cultures at 37 °C in a 95% air/5% CO₂ humidified atmosphere.

Cell Viability: To assess cell viability, cells were seeded in quadruplicate in 96-well plates at a density of 5,000 cells/well for normal bladder urothelial cells; 3,500 cells/well for RT4 and 1,000 cells/well for J82 and UMUC3 in the aforementioned appropriated media for 1 week for normal cells and 24 hours for all other cells. Extracts Broccoli and broccoli sprout GLU and ITC extract treatments were performed at concentrations of 1×10^{-3} , 1×10^{-2} , 5.2×10^{-2} , 0.10, 0.26, 0.52, and 1.0 g of freeze-dried vegetable/L media for 48 hours. Concentrations were obtained by dilution in THF and media with a final THF concentration consistently at 0.125%. ITC extracts for both broccoli and broccoli sprouts were obtained from GLU extracts shaken with excess myrosinase 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% THF for 3 hours. Control cells were treated with the reaction mixture of sodium phosphate buffer and myrosinase. Pure phytochemicals R,S-Sulforaphane, Erucin (LKT laboratories, Inc.) Allyl ITC (Sigma-Aldrich) and Iberin (MP Biomedicals) treatments were performed at 0, 0.5, 1, 2.5, 5, 10, 20 μ M of for 48 hours. Treatments were prepared as stock solutions in DMSO and diluted in culture medium for cell treatments. Control cells were treated with DMSO vehicle at a concentration equal to that of drug-treated cells (final DMSO concentration, $\leq 0.1\%$ by volume). Normal urothelium cells, which divide more slowly than transformed, were exposed to 72 hours of treatment. Cell viability was then measured by SRB assay (Sulforhodamine B Based *In Vitro* Toxicology Assay Kit, Sigma) and percent cell viability was calculated. Experiments were performed in triplicate and data are represented as average \pm standard deviation

Cell Cycle Analysis: Cells were seeded overnight in culture dishes at 1×10^6 /20mL of media for RT4 and 2×10^6 /20mL of media for J82 or UMUC3 cells and treated with 0, 5, 10 or 20 μ M concentrations of sulforaphane or erucin prepared as stock solutions in DMSO and diluted in culture medium for cell treatments for 48h. Cells were harvested by washing with Hanks Balanced Salt Solution (GIBCO), trypsinized and then resuspended in RPMI-1640 medium. Cells were centrifuged, media was removed and cells were washed in cold PBS, and then resuspended in 1mL of cold PBS and resuspended in 10mL of ice-cold 70% ethanol per sample and kept in a -20°C freezer overnight. Samples were then centrifuge, ethanol aspirated and cells resuspended in 1mL of staining buffer (14mL of PBS, 15 μ L 0.1% Triton-X, 300 μ L DNAase-free RNAase A, 750 μ L Propidium Iodide). Samples were kept at room temperature for 30 minutes and then analyzed by flow cytometry using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ) instrument and data analysis using ModFit (Verity Software House, Topsham, ME). For sub-G1 population analysis, both floating and adherent cells were collected. Experiments were performed in quadruplicate and data are represented as average \pm standard deviation

Caspase- 3/7 assay: Cells were seeded in quadruplicate in 96-well white-bottom plates at a density of 3,500 cells/well for RT4 and 1,000 cells/well for J82 and UMUC3 in RPMI-1640 medium for 24 hours. They were then treated for 48 hours with increasing concentrations (0, 5, 10, 20 μ M) of R,S-Sulforaphane or Erucin (LKT laboratories, Inc.) or DMSO vehicle control. Caspase-3/7 activity was assessed by the Apo-ONE Homogenous Caspase-3/7 Assay, Promega. Experiments were performed in triplicate and data are represented as average \pm standard deviation.

Western Blot Analysis: Cells were seeded overnight in culture dishes at 1×10^6 /20mL of media for RT4 and 2×10^6 /20mL of media for J82 or UMUC3 cells and treated with 5, 10 or 20 μM concentrations of Sulforaphane or Erucin diluted in 10% FBS supplemented RPMI 1640 or DMSO vehicle at a concentration equal to that of drug-treated cells (final DMSO concentration, $\leq 0.1\%$ by volume) for 48 hours. Cells were collected and lysed in SDS Lysis buffer (Millipore). Tumor tissue protein extraction was performed using the T-PER Tissue Protein Extraction Reagent (Thermo Scientific) per the manufacturer's protocol. Protein concentrations of the cell and tissue lysates were determined by using the BCA Protein Assay Kit (Thermo Scientific). SDS-PAGE was performed according to manufacturer's protocols provided with NuPAGE pre-cast gels (Invitrogen) and then transferred onto PVDF membranes (Invitrogen) Protein were visualized by chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz). Primary antibodies used were PARP, Survivin and GAPDH (Sigma). Secondary antibodies were purchased from Santa Cruz. Densitometry using the ImageJ (NIH) program was utilized to quantify protein. Experiments were performed in triplicate and data are represented as average \pm standard deviation.

Dietary Preparation for In Vivo Experiment: For the broccoli sprout isothiocyanate extract diet, broccoli sprouts were boiled in water for 30 minutes. Sprouts were filtered out and extract was cooled, exogenous myrosinase added and mixture was incubated for 3.5 hours at 37°C . The mixture was then flash frozen at -40°C and then lyophilized and milled into a fine powder. Total Iberin, Sulforaphane and Erucin were quantified by LC-MS/MS. For the 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. Broccoli ITC extract diet had $197 \mu\text{mol ITC/g}$ extract with 63% SFN, 28% Iberin and 8.4% Erucin. Broccoli

sprout diet had 51 μmol total GLU/ g sprout with 68.5% Glucoraphanin, 21% Glucoiberin and 10.3% Glucoerucin. Broccoli sprout extract was mixed at 2% by weight into AIN 93G diet. Given that mice eat 3g diet/day and weigh on average 25g, they consumed 11.82 μmol total ITCs/day or 473 μmol total ITCs/kg body weight/day (297 $\mu\text{mol}/\text{kg}$ bw SFN, 132 $\mu\text{mol}/\text{kg}$ bw Iberin and 39.7 $\mu\text{mol}/\text{kg}$ bw Erucin). Broccoli sprout diet as mixed at 4% by weight into AIN93G diet, and thus consumed 6.12 μmol total ITCs/day or 245 μmol total ITCs/kg body weight/day (171 $\mu\text{mol}/\text{kg}$ bw glucoraphanin, 51 $\mu\text{mol}/\text{kg}$ bw glucoiberin and 25.21 171 $\mu\text{mol}/\text{kg}$ bw glucoerucin). Diets were stored at $-20\text{ }^{\circ}\text{C}$ and food was changed every other day for the duration of the study (36).

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. Female athymic nude mice (FOXN1^{nu} 4-5 weeks of age), were obtained from Harlan laboratories and acclimated on AIN93G pelleted diet for one 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 flank. Three days after injection, mice were randomized into one of six treatment groups (12mice/group): 1) Vehicle Control Soybean Oil oral gavage once daily 2) 295 $\mu\text{mol}/\text{kg}$ sulforaphane oral gavage once daily 3) 295 $\mu\text{mol}/\text{kg}$ erucin oral gavage once daily 4)Control AIN 93G diet fed *ad libitum* 5) AIN 93 G diet + 4% Freeze-dried broccoli sprout fed *ad libitum* 6) AIN 93 G diet + 2% Freeze-dried broccoli sprout extract fed *ad libitum*. Mice were anesthetized with isoflurane prior to 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

when tumors reached a size of 1cm in either direction, or approximately 2 weeks. At sacrifice, blood was collected and plasma isolated. Tumors were removed and measured and weighed and bladders were also collected, with half of the tissue flash frozen in liquid nitrogen for protein extraction and the other half fixed overnight in 10% neutral buffered formalin and then transferred to 70% ethanol for histological use. Tumor and plasma was also reserved for metabolite quantification studies and metabolites were preserved by the addition of formic acid at a concentration of 0.2%.

Histology: Formalin-fixed paraffin embedded tumor tissue from control and broccoli sprout treated mice were sectioned and stained with Hematoxylin and Eosin (H&E). Ki67 staining was also performed and quantified. Briefly, antigen retrieval was performed by boiling the slides in citrate buffer (Antigen Retrieval Citra Plus, BioGenex, San Ramon, CA) for 15-30 minutes. The subsequent steps were completed in an OptiMax Automated Cell Staining System (BioGenex, San Ramon, CA) at room temperature. Endogenous peroxidase was inhibited by incubation in peroxide blocking solution (Dako Cytomation, Carpinteria, CA) for 5 min followed by 60 min incubation with Ki67 primary antibody (1:50, rat monoclonal antibody, Dako Cytomation), biotinylated secondary antibodies and Vector ABC Elite kit (Vector Laboratories, Burlingame, CA) were then used. Color was developed by 10 min incubation with DAB chromogen solution (Vector Laboratories). Slides were counterstained with Mayers hematoxylin for 2 min and mounted. The percentage of positive nuclei was then calculated based on the following formula: labeling index (%) = $L / (L+C) \times 100$, where L= labeled cells and C = counterstained, unlabeled cells.

Metabolite Quantification: Mercapturic acid pathway metabolites of sulforaphane and erucin were quantified by UPLC-MS/MS. Plasma proteins were first precipitated with cold trifluoroacetic acid and 10uL of supernatant injected on a reversed phase UPLC column. ITC metabolites were separated with a gradient of 0.1%formic acid versus 0.1%formic acid in acetonitrile and eluate interfaced with an electrospray probe to a triple quadrupole mass spectrometer in positive ion mode. Respective molecular ions were fragmented by collision induced dissociation to characteristic fragment ions for detection. Calibration curves were prepared for SFN and ECN conjugated to glutathione, cysteinyl glycine, cysteinyl or N-acetyl cysteine as well as free SFN.

UPLC-MS/MS of ITC Metabolites: To 100uL mouse blood plasmas on ice was added cold trifluoroacetic acid to 20%(v/v) to precipitate proteins. Samples were centrifuged briefly and supernatant collected for injection on a UPLC-MS/MS system. A 0.1%formic acid/0.1%formic acid in acetonitrile (A/B) mobile phase system was used to separate the metabolites. The initial condition was 0%B increased to 10%B over 1min, 10-33.3%B over 1.5min, 33.3-72%B over 1.5min with 2 min for re-equilibration. The eluent was interfaced with a triple quadrupole mass spectrometer via an electrospray probe in positive polarity. Each metabolite was monitored as an MS/MS transition (parent>daughter ion) using collision induced dissociation. Standards of ITC metabolites and free ITCs were purchased from Toronto Research Chemicals except for CysGly SFN and the erucin metabolites which were produced by reacting SFN or ERN isothiocyanates with their respective conjugating group (glutathione, cysteinyl glycine, cysteine or N-acetyl cysteine) and isolating them by preparative chromatography.

Statistics: Statistical significant was tested by two-tailed Student's t-test or ANOVA, followed by the Student-Newman-Keuls multiple comparisons test, using InStat software (GraphPad). Significance was set at $p \leq 0.05$.

Results

Quantification of glucosinolates in broccoli and broccoli sprout reveals broccoli sprouts have significantly higher levels than mature broccoli

In order for *in vitro* and *in vivo* studies utilizing vegetable extracts to be meaningful, it is important to characterize the active phytochemicals present in these extracts, especially given their great variation found among plants. Given this, we utilized our previously developed LC-MS/MS method (37) to identify and quantify individual, intact glucosinolates (GLUs) in broccoli and broccoli sprouts (**Table 1**). We then hydrolyzed these GLU extracts into isothiocyanates (ITCs) using exogenous myrosinase and then quantified total ITCs utilizing a cyclocondensation reaction (**Table 2**). Significantly higher levels of total combined GLUs were detected in the broccoli and broccoli sprout extracts, 13.3 μmol and 84.6 μmol respectively. Glucoraphanin, the precursor to sulforaphane, was the predominant GLU found in both broccoli and broccoli sprouts, making up 64.4% and 52.6% of the total GLU composition, respectively. Aliphatic GLUs glucoiberin and glucoerucin, precursors of iberin and erucin, were the second and third highest GLUs in broccoli sprouts and indole GLUs glucobrassicin, precursor to indole-3-carbinol and neoglucobrassicin were the second and third predominant GLUs in broccoli (**Table 1**). As predicted, the amount of total converted isothiocyanates was significantly higher in broccoli sprouts over broccoli (**Table 2**).

Isothiocyanates extracts and pure phytochemicals significantly inhibit superficial and invasive human bladder cancer cell line viability

We first wanted to test the potential biological activity of isothiocyanates in bladder cancer by testing whether broccoli and broccoli sprout glucosinolate and isothiocyanate extracts can inhibit superficial (RT4) and invasive (J82) human bladder cancer cell viability. We chose to utilize a Sulforhodamine B assay (SRB) over the MTT or MTS assay due to the fact that these compounds have been shown to have antioxidant properties and the MTT/MTS assays are based on reductase enzyme activity while the SRB assay is based on staining cells and looking at total cell bulk, giving more accurate results. All treatments were performed for 48 hours. We found that glucosinolate extracts, from either broccoli or broccoli extracts, did not have an effect on cell growth on either cell line. However, ITC extracts caused a significant dose-dependent decrease in cell viability of both RT4 and J82 cell lines. Interestingly, mirroring our quantification data which shows that broccoli sprouts have significantly higher concentrations of ITCs, we see a significantly greater dose-dependent decrease in cell viability of both RT4 and J82 cells when treated with broccoli sprout ITC extract versus broccoli ITC extract (**Fig 1A**). We next wanted to determine of all isothiocyanates found in broccoli and broccoli sprouts, which is the most efficacious in inhibiting our bladder cancer cell lines. We chose to R,S Sulforaphane over purely R or S sulforaphane because our studies show that there is no significant effect of inhibition of cell viability of bladder cancer cell lines between these enantiomers. We found that in both superficial (RT4) and invasive (J82, UMUC3) cell lines, sulforaphane is the most potent inhibitor ($IC_{50} = 11.5 \mu\text{M}$ in RT4 cells and $IC_{50} = 5.8 \mu\text{M}$ in UMUC3 cells for 48 hours treatment). Erucin was the next compound with greatest efficacy ($IC_{50} = 15.8 \mu\text{M}$ in RT4 cells and $IC_{50} = 9.2 \mu\text{M}$ in UMUC3 cells for 48 hours treatment). This is followed by Allyl ITC and

then Iberin (**Fig 1B**). We then compared a panel of human bladder cell lines ranging from normal human urothelial cells (Normal HU) to superficial (RT4) to invasive (J82, UMUC3). We found that normal human urothelial cells are least sensitive to the inhibitory effects of sulforaphane and erucin, followed by superficial cells (RT4) and finally by invasive cells (J82 and UMUC3- J82 data not shown) (**Fig 1C**). In summary, we found that broccoli and broccoli sprout glucosinolate extracts do not inhibit bladder cancer cell viability, while broccoli and broccoli sprout isothiocyanates do, with broccoli sprouts isothiocyanate extracts causing significantly greater inhibition due to their higher levels of isothiocyanates. Of all isothiocyanates, sulforaphane and erucin are the most potent inhibitors and normal human urothelial cells are least sensitive to these inhibitory effects, followed by superficial and then invasive.

Sulforaphane and erucin treatment causes human bladder cancer cells to accumulate in the G2/M phase of the cell cycle

After determining that isothiocyanates have the ability to inhibit the viability of bladder cancer cell lines, we wished to further analyze how this inhibition is occurring. We first analyzed the effects of isothiocyanates sulforaphane and erucin on modulating the cell cycle of bladder cancer cells. We treated both non-invasive (RT4) and invasive (J82, UMUC3 – UMUC3 data not shown) cells with either vehicle control (DMSO), 5, 10 and 20 SFN or ECN μ M for 48 hours and observed the status of cells in the cell cycle after treatment. We observed a significant, dose-dependent accumulation of cells in the G2/M phase. The S phase accumulation was not significantly altered. SFN and ECN altered the cell cycle in a similar way and to a comparable extent (**Fig 2**).

Apoptosis is significantly induced when bladder cancer cells are treated with sulforaphane and erucin

To further evaluate how isothiocyanates can inhibit the viability of bladder cancer cell lines, we studied whether these compounds can induce apoptosis. We utilized a caspase 3/7 activity assay and also looked at PARP, one of the cleaved proteins of caspase 3/7 enzymes. We also looked at expression of survivin, an important anti-apoptotic protein and a good predictor of bladder cancer disease progression (38). Upon treatment with SFN or ECN for 48hours, in both non-invasive (RT4) and invasive (J82, UMUC3) cells, we saw a significant dose-dependent increase in caspase 3/7 activity (**Fig 3A**). We also observed a significant dose-dependent induction of PARP cleavage and a dose-dependent decrease of survivin expression (**Fig 3B**). Together this data strongly suggests that broccoli and broccoli sprout isothiocyanates, particularly sulforaphane and erucin, can significantly inhibit both superficial and invasive bladder cancer cell viability and that this inhibition is due to accumulation of cells in the G2/M phases of the cell cycle as well as the induction of apoptosis.

Broccoli sprout isothiocyanates significantly inhibit bladder cancer and induce apoptosis in vivo

After determining the inhibitory effects of broccoli isothiocyanates *in vitro*, we wanted to translate our work into an *in vivo* xenograft model. We injected female athymic nude mice with UMUC3, invasive human bladder cancer cells and exposed them to one of six treatments: Control AIN93G diet, 2% broccoli sprout isothiocyanate extract diet, 4% broccoli sprout diet, Control soybean oil gavage, 295 $\mu\text{mol/kg}$ body weight sulforaphane gavage and 295 $\mu\text{mol/kg}$ body weight erucin gavage. Before being incorporated into the diet, broccoli sprout GLUs and

broccoli sprout extract ITCs were quantified by UPLC/MS-MS. We wished to compare how a dietary administration versus a pure compound treatment would effect tumor growth. We utilized previously published work using broccoli sprout extract at 160 $\mu\text{mol/kg}$ body weight/day in a bladder cancer model in rats to help guide us in determining what concentration of broccoli sprout isothiocyanates to utilize (24). Given previous findings that rats absorb isothiocyanates more efficiently than mice, and to best match total sulforaphane in our broccoli sprout extract diet (297 $\mu\text{mol/kg}$ bw/day) we decided to sulforaphane at 296 $\mu\text{mol/kg}$ bw/day in our study (39). Encouraged by our *in vitro* data, we matched this concentration in our erucin treatment to compare the efficacy of erucin to sulforaphane *in vivo*. We matched to the best of our ability, total glucosinolate content in the broccoli sprout diet with total isothiocyanate content in the broccoli sprout isothiocyanate without making the percentage of broccoli sprout in our diet unreasonable and not practical for incorporation into human diets in the future, thus we chose 4% over 8% (giving a total ITC concentration of 245 μmol GLU/ kg bw/day containing 172 $\mu\text{mol/kg}$ bw/day of glucoraphanin, the precursor of sulforaphane). Mice were treated for two weeks, where mouse weight and tumor growth rate was monitored, and were then sacrificed, and tumors harvested and weighed. We saw a significant inhibition (40% or greater) of tumor weight in all treated groups, with the most significant inhibition seen in the erucin treated group. There was no significant change in mouse weight through the study (data not shown). Interestingly, broccoli sprout and broccoli sprout isothiocyanate extract groups exhibited similar inhibition. Growth rate was also inhibited in all groups following similar trends to the final tumor weight data (**Fig 4A**). We next performed Ki67 staining, to determine how cell proliferation was effected by broccoli sprout diet treatment. We saw a significant decrease in % positive cells stained with Ki67, suggesting that cell proliferation was significantly inhibition with broccoli

sprout treatment. (**Fig 4B**). In addition, we wished to test our *in vitro* findings of an induction of apoptosis with broccoli isothiocyanate treatments *in vivo*, and thus we looked at PARP cleavage, an indication of caspase 3/7 activity and saw a significant increase in cleaved PARP protein in broccoli sprout treated group when compared to control (**Fig 4B**). These data further support the ability of broccoli isothiocyanates to inhibit bladder cancer.

Isothiocyanates administered by diet or pure gavage are efficiently absorbed into the blood

We found metabolites of sulforaphane and erucin were absent in control mice and present in plasma of all treated groups, with dietary and pure phytochemical treatments resulting in similar plasma metabolite concentrations (micromolar range). There was also significant absorption of broccoli isothiocyanates in tumor and bladder tissue. Interestingly, broccoli sprout diets lead to very similar plasma concentrations when compared to hydrolyzed broccoli sprout ITC extracts (**Fig 5**). N-acetylcysteine conjugates were found at highest concentrations, followed by cysteine and glutathione conjugates and small amounts of detectable cysteinylglycine conjugates and free sfn. Interestingly, we observed a robust interconversion of sulforaphane and erucin, as both metabolites were present in the blood of mice fed pure individual compounds.

Discussion

The goal of our study was to determine if broccoli isothiocyanates have the ability to inhibit bladder cancer *in vitro* and *in vivo* and to determine the metabolism and bioavailability of these compounds in tumor and bladder tissue. There have been a handful of studies looking at the ability of isothiocyanates to modulate bladder carcinogenesis *in vivo*. One of the most recent looked at the effect of allyl isothiocyanate rich mustard seed powder (71.5 mg/kg giving from a

singirin dose of 9 $\mu\text{mol/kg}$) in an orthotopic rat bladder cancer model and found a 34.5% inhibition of bladder cancer growth and blocked muscle invasion (40). Another study showed that sulforaphane can inhibit 4-aminobiphenyl-induced DNA damage in RT4 cells and in bladder tissue (41). In a long term (36 week) rat study, animals were fed 40 or 160 $\mu\text{mol/kg bw/day}$ broccoli sprout extract and were given 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in the drinking water, a specific bladder carcinogen which produces bladder cancer which histologically resembles human bladder cancer. A significant decrease in incidence, multiplicity, size and progression of bladder cancer was observed in the broccoli extract treated group, particularly at the 160 $\mu\text{mol/kg}$ concentration. This was partly attributed to the broccoli extract's ability to significantly induce glutathione-S-transferase and NAD(P)H: quinone oxidoreductase 1, enzymes that detoxify oxidants and carcinogens (24). The uniqueness of our finding is that we were able to show that this inhibition is independent of the ability of isothiocyanates to detoxify carcinogens and prevent DNA damage (24, 41) due to the fact that the cancer cells were already established in a xenograft model and our model was carcinogen induced. This suggests that other mechanisms of action are at play which gives isothiocyanates the ability to have an anti-bladder cancer effects.

The amount of GLUs in both the broccoli and broccoli sprout extracts was consistent with that found in the literature (**Table 1 and 2**) (36). Generally, mature plants have fewer GLUs than immature plants because the GLUs are both hydrolyzed by myrosinase or diluted as the plant grows (42). The mechanism for indole GLUs' increase as the plant matures is still unknown, but it has previously been estimated that there are 50% more indole GLUs in mature broccoli than immature (17). In our cell viability data with broccoli and broccoli sprout GLU and

ITC extracts, where ITC extracts were made by adding exogenous myrosinase to the GLU extracts, it was apparent that GLU do not have an effect on bladder cancer cell viability while ITCs do, providing strong evidence for the activity of ITCs in bladder cancer (**Fig 1A**). This may at least partly explain epidemiological observation of increased broccoli consumption correlating with decreased bladder cancer risk (12, 43). We showed that sulforaphane and erucin can both induce significant accumulation of bladder cancer cells in the G2/M phase (**Fig 2**). This mirrors previous findings which shows that broccoli sprout extract can induce cell cycle arrest in the G2/M and S phase through downregulation of Cdc25c and disruption of the mitotic spindle (22). For the first time, we were able to show that not only do broccoli sprout isothiocyanate extract and sulforaphane have the ability to inhibit bladder cancer *in vivo*, but broccoli sprouts and erucin also have this ability with comparable or even greater efficacy (**Fig 4**). Our 4% broccoli sprout diet had half the amount of total precursor glucosinolates than our 2% broccoli sprout ITC extract diet, however, the inhibitory effects seen were comparable. This could suggest that either there is something in the broccoli sprouts that was not present in the broccoli sprout isothiocyanate extract that may be exerting inhibitory effects, working with the converted isothiocyanates, or it could also suggest that the total amount of absorbed isothiocyanates was saturated and giving a higher dose of ITCs did not exert a more potent effect (**Fig 4**). Our metabolite data suggests the latter possibility due to the fact that broccoli sprout ITC extract diet and broccoli sprout diet had similar total metabolite levels (**Fig 5**). Erucin showed strong activity, having comparable or greater efficacy than sulforaphane in the inhibition of bladder cancer cell viability, modulation the cell cycle, induction of apoptosis and inhibiting bladder tumor growth (**Fig 2-4**). Erucin was also well absorbed in the plasma (**Fig 5**). Given our observation of the robust interconversion of sulforaphane to erucin and vice versa *in vivo* it is

interesting to consider which of these compounds are inducing these effects (**Fig 5**). We showed that normal cells are less sensitive to the inhibitory effects of these compounds compared to superficial and invasive human bladder cancer cells. In addition, treatment with isothiocyanates did not show any toxicity, organs were of normal size and histology and there was no significant difference in mouse weight throughout the study (data not shown).

Isothiocyanates have also been shown to have bladder cancer inducing properties, particularly phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC). Both pre- and post- initiation events were studied. Pre-initiation, 0.1% PEITC or BITC for 14 days lead to increased inflammatory cell infiltration and hyperplasia of the bladder epithelium (44). In addition, post-initiation effects of 0.1 % PEITC and BITC were examined in urinary bladder carcinogenesis with or without pre-treatment with diethylnitrosamine (DEN) and BBN, and both isothiocyanates showed strong promoter and some complete carcinogenic potential of bladder cancer (45). One possible reason for this observation could be the accumulated dose of urinary ITCs with this dosing regimen. It is estimated that NAC-ITCs could have been constantly maintained at levels far greater than 1 mmol/L and that although ITCs can be beneficial at preventing or inhibiting bladder cancer growth at lower doses, at excessively higher doses, they may be harmful (21). In addition, it is possible that the benzyl or phenethyl isothiocyanates may have some harmful effects but not other ITCs such as sulforaphane or erucin. Broccoli and broccoli sprouts do not have appreciable levels of these isothiocyanates and may be a better option over cruciferous vegetables containing higher levels of PEITC or BITC such as cauliflower, cabbage or Brussels sprouts, especially if these compounds are used in high doses for prevention of treatment of bladder cancer (46). In our study we showed that 295 $\mu\text{mol/kg}$ bw/day of either sulforaphane or erucin, or 2% broccoli sprout extract (total ITCs = 473 $\mu\text{mol/kg}$

bw/day) or 4% broccoli sprout diet (total GLUs = 245 $\mu\text{mol/kg}$ bw/day) was both safe and efficacious at inhibiting bladder cancer (**Fig 4**), at least in a two week time span. It would be beneficial to test these levels of isothiocyanates in a long term transgenic bladder cancer model to test the ability of these compounds to inhibit non-carcinogen induced bladder cancer, without inducing long term toxic effects (47-52).

There are several potential preventative approaches which can be employed in humans if broccoli shows strong promise in inhibiting bladder cancer pre-clinically. One means would be through a primary prevention strategy. Here, recommendations to the general public or to individuals at higher risk, such as smokers or those with occupational exposures, can be used to advise individuals to more frequently consume cruciferous vegetables to help prevent the development of bladder cancer. Another approach is a secondary prevention strategy, where patients who have already undergone surgical resection of non-invasive cancer would be given cruciferous vegetables, through dietary (foods and novel food products) or perhaps pharmacologic products, in order to help prevent recurrence of their disease. This could significantly contribute to lowering health care costs, which are high due to monitoring (cystoscopic exams) and treating these patients (intravesical immunotherapy and chemotherapy), as well as help alleviate patient suffering. Our future hope for these compounds is to use these in a secondary chemoprevention approach to help ameliorate the bladder cancer burden.

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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	0	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	0	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

Table 1: Characterization of glucosinolates in broccoli and broccoli sprout extracts, as quantified by HPLC-MS/MS. Broccoli sprouts have significantly higher levels of total glucosinolates than broccoli. Values represent the mean of two samples.

Vegetable	Total non-indole GLUs (μmol/g) *	Total ITCs (μmol/g) *	% GLU converted to ITC
Broccoli	8.78	6.65 ± 0.65	75.7 ± 0.9
Broccoli Sprout	80.6	96.0 ± 13.1	119 ± 11

Table 2: Amount of total combined non-indole GLUs, ITCs, and percent conversion of GLUs to ITCs in broccoli and broccoli sprout extracts. Values represent the mean ± SD (n=4).

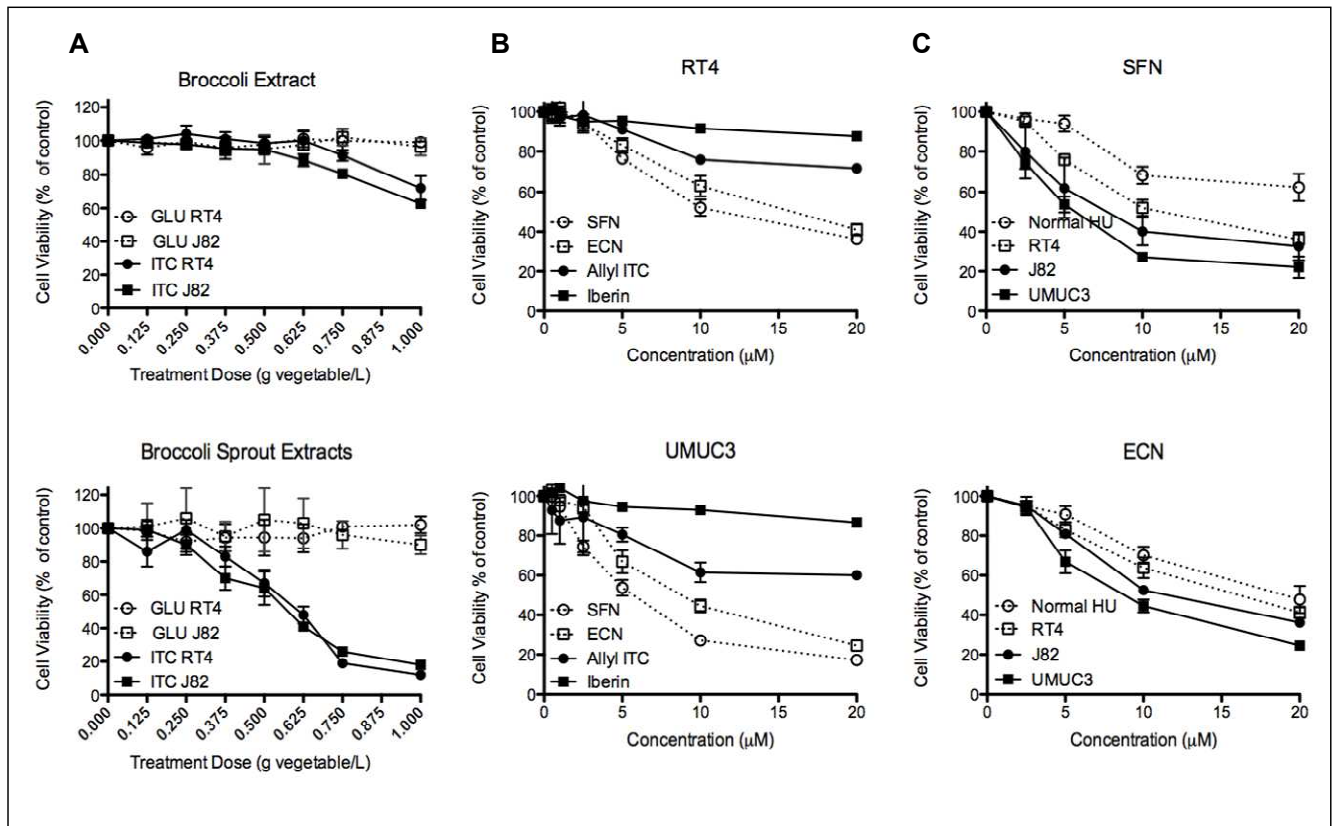


Figure 1: Broccoli and broccoli sprout isothiocyanates significantly inhibit human bladder cancer cell lines. Growth inhibitory effects of broccoli and broccoli sprout glucosinolates and isothiocyanates were assessed by Sulforhodamine B assay (SRB), 48 hours of treatment. *A.* The effect of glucosinolates and enzymatically-hydrolyzed glucosinolates (isothiocyanates) extracted from broccoli and broccoli sprouts on the cell viability of RT4 (superficial) and J82 (invasive) human bladder cancer cell lines. Percent cell viability was calculated by (absorbance of the treatment wells x 100) / absorbance of the control. *B.* Isothiocyanates found in broccoli and broccoli sprout extracts (sulforaphane, erucin, allyl ITC, iberin) were compared for efficacy in RT4 (superficial) and UMUC3 (invasive) cell lines. *C.* Sulforaphane and Erucin treatment was compared in a panel of human bladder cells ranging from normal human urothelial (Normal HU), superficial bladder cancer (RT4) and invasive (J82, UMUC3). Data represents the means of three independent experiments with error bars representing standard deviations.

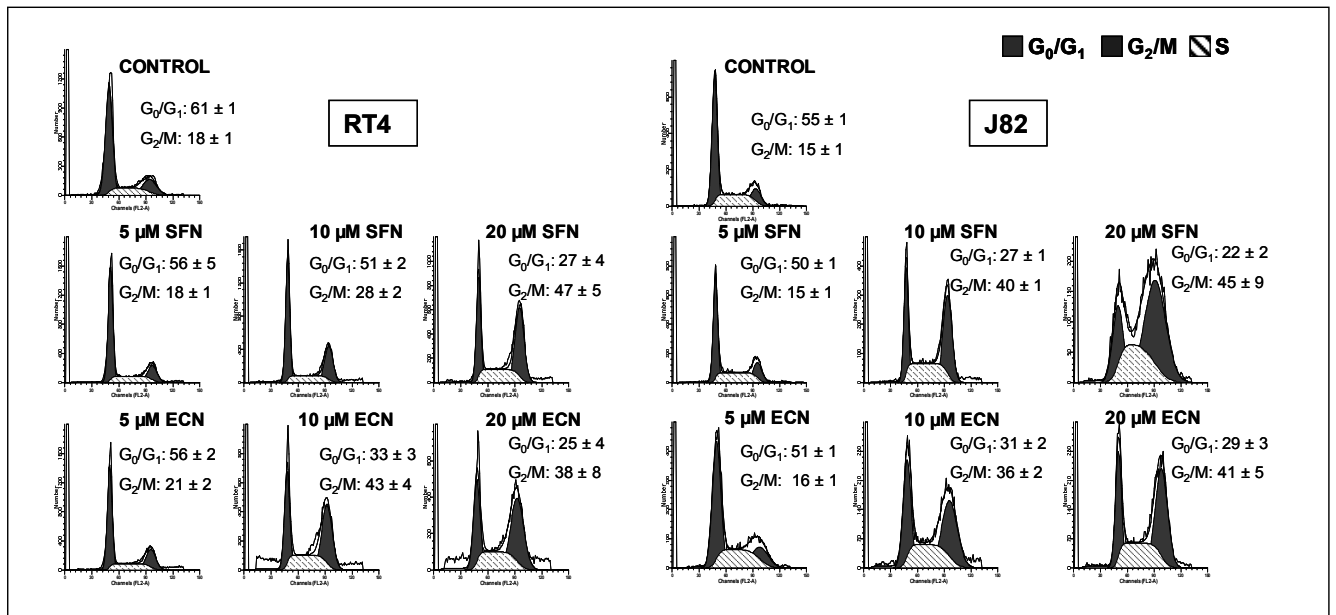


Figure 2: Sulforaphane and erucin treatment leads to cell cycle alteration of human bladder cancer cells. RT4 (superficial) and J82 (invasive) human bladder cancer cells were treated with vehicle control (DMSO) or 5, 10 and 20 μM of sulforaphane (SFN) or erucin (ECN) for 48 hours and then stained with propidium iodide for cell cycle analysis through flow cytometry. Data represents the means of four independent experiments ± SD.

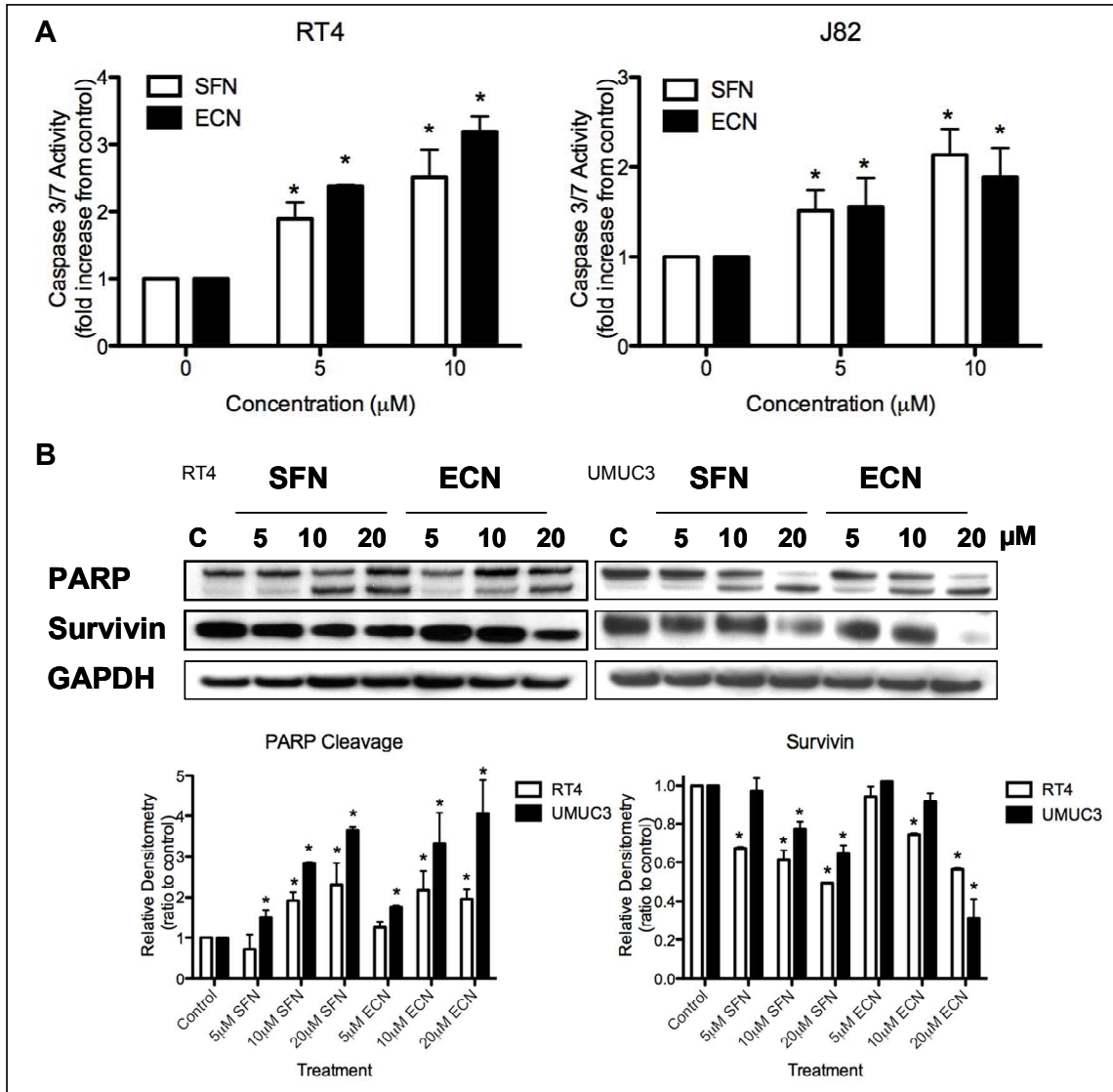


Figure 3: Sulforaphane and erucin treatment causes apoptosis in human bladder cancer

cell lines. Superficial (RT4) and Invasive (J82, UMUC3) bladder cancer cells were treated with

increasing doses of sulforaphane (SFN) or erucin (ECN) for 48 hours *A*. Caspase 3/7 activity was assessed through Promega Apo-ONE® Homogenous Caspase 3/7 fluorescence assay. *B*.

Cleavage of PARP, an indicator of apoptosis, and expression of survivin, an antiapoptotic protein was assessed through western blot analysis and quantified through densitometry. Data represents

the means of three independent experiments with error bars representing standard deviations

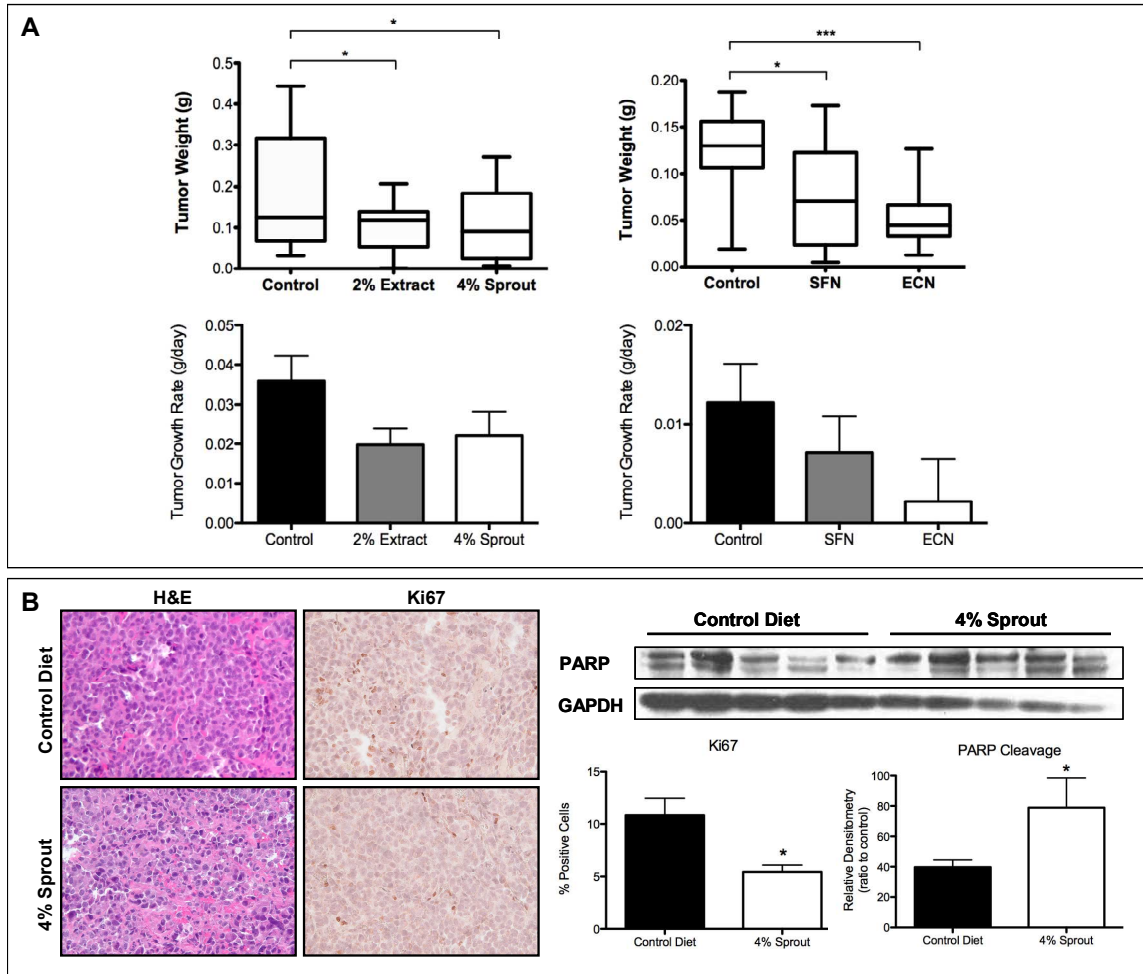


Figure 4: Broccoli isothiocyanates cause inhibition of bladder cancer and induction of apoptosis *in vivo*. Female athymic nude mice were injected with UMUC3 (invasive) human bladder cancer cells in each flank (2 tumors per mice) and treated with either Vehicle Control Soybean Oil oral gavage once daily 2) 295 $\mu\text{mol/kg}$ sulforaphane oral gavage once daily 3) 295 $\mu\text{mol/kg}$ erucin oral gavage once daily 4) Control AIN 93G diet fed *ad libitum* 5) AIN 93 G diet + 4% Freeze-dried broccoli sprout fed *ad libitum* 6) AIN 93 G diet + 2% Freeze-dried broccoli sprout extract fed *ad libitum*. Treatments were administered for 2 weeks, before tumors could reach greater than 1cm in any direction *A*. At sacrifice, tumors were harvested and weighed and final tumor weights were compared between groups. Tumor growth rates were also monitored during the time of treatment and compared. Data represents box plots of median and min and

max values with * $p < 0.05$, *** $p < 0.001$ for tumor weights and mean with error bars representing standard error. *B.* Tumors from control diet and broccoli sprout diet treated mice were fixed, embedded, sectioned and stained with hematoxylin and eosin (H&E) or Ki67 with background hematoxylin stain, a marker of proliferation. Ki67 was quantified as percent positive cells. Cleaved PARP was compared in control versus broccoli sprout treated groups and quantified. Data represents five independent samples and error bars represent standard deviation.

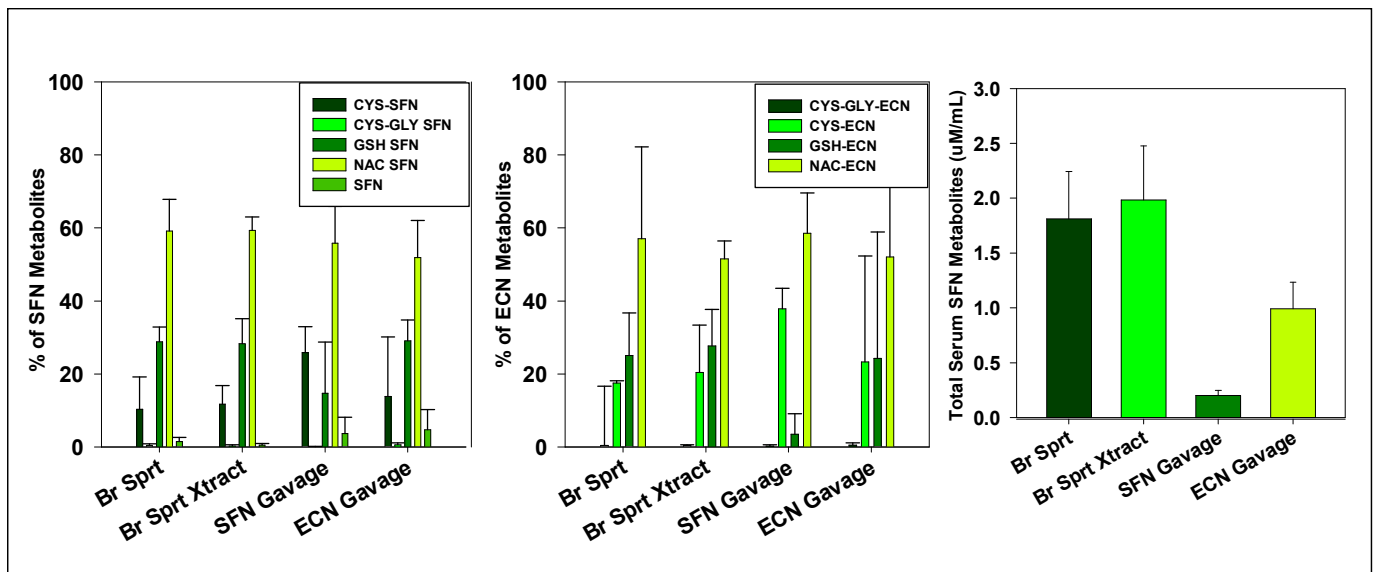


Figure 5. Profiles of SFN and ECN metabolites in mouse plasmas from 2%BSx (broccoli sprout glucosinolates converted to ITC), 4%fd BS (freeze dried broccoli sprouts), sfn gav (gavage with SFN in soybean oil), ecn gav (gavage with erucin in soybean oil) and total ITC metabolite levels per group.