BROCCOLI BIOACTIVES:
IMPACT ON ENZYME INDUCTION, ESTROGEN METABOLISM AND HUMAN CANCER CELL GROWTH

BY

SONJA E. VOLKER

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Nutritional Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Doctoral Committee

Professor John W. Erdman, Jr., Chair
Professor Elizabeth H. Jeffery, Director of Research
Professor Janice M. Bahr
Professor Alan M. Diamond, University of Illinois at Chicago

ABSTRACT

Estrogen-associated cancers are a leading cause of mortality and morbidity in U.S. men and women. Consumption of cruciferous vegetables, such as broccoli, cauliflower and cabbage, is associated with reduced risk for these and other cancers. The chemoprotective properties of crucifers are traditionally attributed to high levels of glucosinolates and their bioactive breakdown products.

A proposed mechanism of protection against estrogen-associated cancers by cruciferous vegetables is alteration of estrogen metabolism toward formation of less estrogenic metabolites. Supplementation with the glucosinolate breakdown product indole-3-carbinol (I3C) has been shown to increase formation of 2-hydroxyestrogens, estrogen metabolites with weak estrogenic activity. However, the observed reduction in risk for estrogen-associated cancers with increased consumption of cruciferous vegetables may relate to increased formation of anticarcinogenic 2-methoxyestrogens derived from further metabolism of 2-hydroxyestrogens.

In this study, glucosinolate breakdown products found in broccoli enhanced formation of 2-methoxyestradiol from the parent compound 17β-estradiol *in vitro* and *ex vivo*, mainly via upregulation of the phase I detoxification enzyme cytochrome P450 (CYP) 1A. Broccoli inhibited growth of human prostate and ovarian cancer cells via induction of apoptosis, but this was not due to formation of 2-methoxyestradiol. Compared to the purified glucosinolate breakdown products I3C and sulforaphane (SF), broccoli inhibited cancer cell growth to a greater extent.

Both SF and the flavonols quercetin and kaempferol have been shown individually to upregulate activity of the phase II detoxification enzyme NAD(P)H dehydrogenase, quinone 1 (NQO1). The present study demonstrated that combinations of purified SF, quercetin and kaempferol increased NQO1 activity in a synergistic manner in murine hepatoma cells.

Broccoli is an active accumulator of selenium, and the concentration of this mineral in the plant tissue may be orders of magnitude greater than that in the soil. Selenium enrichment has been shown to enhance the anticarcinogenic property of broccoli. An underlying mechanism may be upregulation of CYP1A and NQO1 activities, which may result in enhanced inactivation and subsequent excretion of potentially carcinogenic compounds. In this study, selenium enrichment resulted in increased levels of the indole glucosinolate neoglucobrassicin in the plant tissue, and a concomitant increase in CYP1A and NQO1 activities *in vitro*. Breakdown products of neoglucobrassicin, but not selenium, appear to account for the observed increase in CYP1A activity; their effect on NQO1 activity remains to be determined.

Taken together, these results show that I3C and SF, the main bioactive glucosinolate breakdown products derived from broccoli, are not solely responsible for the anticancer effects of broccoli. Rather, other bioactive compounds present in the vegetable, alone or in combination, may contribute to the chemopreventive potential of the vegetable.

TABLE OF CONTENTS

CHAPTER 1

Introduction

Estrogen-associated cancers are a leading cause of mortality and morbidity in U.S. men and women. For 2010, an estimated 39% of all new cancer cases and 24% of all cancer deaths in women were attributed to cancers of the breast, ovary and uterus. In men, prostate cancer accounted for 28% of all new male cancer cases and 11% of all male cancer deaths (1). Comparable incidence patterns are found in other western societies, suggesting that environmental factors, including nutrition, play an important role in the development of the disease (2).

The overall incidence of cancer may be reduced significantly by adopting an appropriate diet rich in plant-based foods (3). This assumption is reflected in the American Cancer Society Guidelines on Nutrition and Physical Activity for Cancer Prevention, which emphasize consumption of fruits, vegetables and whole grains (4). Of particular interest are the cruciferous vegetables, such as broccoli, cauliflower and cabbage. Compared to all fruits and vegetables, consumption of crucifers is associated with reduced risk for various cancers, including those influenced by estrogen status (5-7). This enhanced chemoprotective effect of crucifers is traditionally attributed to high levels of glucosinolates and their bioactive breakdown products (5, 8).

A proposed mechanism of protection against estrogen-associated cancers by cruciferous vegetables is alteration of estrogen metabolism toward formation of less estrogenic metabolites (9). Supplementation with the broccoli glucosinolate breakdown product indole-3-carbinol (I3C) has been shown to increase formation of 2-

hydroxyestrogens with relatively weak estrogenic activity compared to the alternative metabolite 16α-hydroxyestrogen (10), and this change in metabolism is associated with lower risk for cancer of the breast (11, 12), ovary (13) and prostate (14). However, accumulation of 2-hydroxyestrogens is not necessarily beneficial, since this metabolite could undergo subsequent oxidation to highly reactive estrogen quinones and lead to formation of stable DNA adducts, which may initiate carcinogenesis (15-17). 2-Hydroxyestrogens can also be methylated to 2-methoxyestrogens (18). In particular, 2-methoxyestradiol has emerged as a promising anticancer agent and is currently being evaluated in phase I and II clinical trials (19, 20). Presently, the relationship between glucosinolate breakdown products found in broccoli and formation of 2-methoxyestradiol is essentially unknown.

Selenium-enriched broccoli has been shown to further enhance activity of the detoxification enzymes cytochrome P450 (CYP) 1A and NAD(P)H dehydrogenase, quinone 1 (NQO1) (21), which may, in part, be responsible for its greater chemoprotective effect compared to standard broccoli. Besides increasing the levels of selenium in the plant tissue, enrichment altered the glucosinolate profile of the plant (21). Whether the increase in detoxification enzyme activity is directly due to higher concentrations of selenium in the plant, or a result of changes in the glucosinolate content remain to be determined.

The overarching goal of this study was to determine the effect of broccoli bioactives, either as purified compounds or as a mixture derived from the whole food, on formation of anticarcinogenic 2-methoxyestradiol, and on human prostate and ovarian cancer cells. Possible interactive effects between purified compounds on cancer

cell growth and detoxification enzyme activity were also evaluated, as well as the impact of selenium-enrichment on the bioactivity of broccoli. The results of this study will provide further insight into which bioactives are responsible for the anticancer effects of broccoli, and contribute to the development of broccoli with enhanced health benefit through enrichment with bioactive components.

CHAPTER 2

Literature review[1]

2.1. Estrogen-associated cancers

Estrogens are involved in numerous biological processes including female reproductive development, bone formation and maintenance, cardiovascular and neurological health (22). However, prolonged and cumulative exposure to estrogens, as a result of early menarche, late menopause, absence of pregnancies or late first pregnancy, is thought to increase risk for various cancers, including those of the breast, ovary and uterus (23-27). In the United States, estrogen-associated cancers are a leading cause of morbidity and mortality in women. In 2010, cancers of the breast, ovary and uterus accounted for 39% of all new cancer cases in women, and for 24% of all cancer deaths in women. Of these cancers, cancer of the breast was the most frequently diagnosed (28% of all new female cancer cases), but had the highest overall five-year survival rate (89%). This was in stark contrast to ovarian cancer: although women were less likely to be diagnosed with the disease (3% of all new female cancer cases), the overall five-year survival rate was less than 50%, thus making ovarian cancer the deadliest gynecological malignancy (1, 28).

Prostate cancer is considered an androgen-associated disease; however, estrogens are thought to play an important role in the development of the disease as well. Estrogens have been shown to increase growth of prostate cancer cells *in vitro* (29), and enhance androgen-induced prostate cancer in an animal model (26).

[1] Figure 2.4. is reproduced, with permission of Annual Reviews, from Halkier BA, Gershenzon J. Biology and biochemistry of glucosinolates. Annu Rev Plant Biol. 2006;57:303-33.

Compared to white men, African-American men were shown to have an increased risk for prostate cancer (28). Serum estrogen levels were found to be significantly higher in African-American men in comparison to their white American counterparts (30). Circulating androgen levels, but not estrogen levels, were shown to decrease with increased age (31), resulting in a relative estrogen-dominant environment in the aging male. Taken together, these observations support the hypothesis that estrogens are a contributing factor in the development of prostate cancer.

Prostate cancer is the most frequently diagnosed male cancer and the second leading cause of male cancer deaths. In 2010, prostate cancer made up 28% of all new male cancer cases and 11% of all male cancer deaths. Despite these high rankings, the overall five-year survival rate for prostate cancer was nearly 100% (1, 28).

The differences in the overall five-year survival rate between ovarian (less than 50%) and prostate cancer (nearly 100%) are in part due to the stage of the disease at the time of diagnosis: whereas 81% of all prostate cancer cases are diagnosed while the cancer is still confined to the primary site, 62% of ovarian cancer cases are diagnosed after the cancer had already spread to distant sites of the body. The five-year survival rate for prostate cancer drops to less than 30% if the disease has already metastasized; the five-year survival rate for women with localized ovarian cancer is 92.5% (28). These numbers emphasize the importance of early cancer detection and the need for effective, reliable screening methods.

2.2. Diet and cancer

Inherited genetic factors have long been viewed as the key determinants of cancer development. One of the first lines of evidence indicating that non-genetic factors could also play a role in the development of the disease came from the observed dramatic increase in malignant lung tumors among smokers in the early twentieth century (32). The variation in global cancer incidence between Western and non-Western societies (Fig. 2.1) also suggests that non-genetic factors are involved in cancer development (33). While some of this variation can be explained by differences in cancer screening and diagnosis between Western and non-Western societies, the idea that non-genetic factors may contribute to cancer development is further supported by studies comparing patterns of cancer incidence between native-born and immigrant populations. These studies have consistently shown that whereas cancer incidence patterns of first-generation immigrants resemble those of their native country, subsequent generations tend to acquire the patters of their new country. Adoption of new incidence patterns was found to be particularly strong for estrogen-associated cancers in migrants moving from developing to developed societies (34-36). For women living in Western societies, the risk of developing an estrogen-associated cancer (of the breast, ovary or uterus) can be more than 2.5-fold greater compared to the risk for women living in non-Western societies (Fig. 2.2). Risk of prostate cancer can vary even more dramatically between different societies: Western men have a nearly 20-fold increased chance of developing the disease compared to non-Western men (Fig. 2.3). These observations show that risk for cancer, including those associated with estrogen status, is not determined by genetic factors alone, but also by external factors, such as

nutrition. Thus, changes in dietary patterns may alter the incidence rate for these diseases.

The observed differences in global cancer incidence and between immigrants and their subsequent generations formed the basis of a landmark study by Doll and Peto that highlighted the role of non-genetic factors, including environmental and lifestyle factors such as diet, in the development of cancer. One of the conclusions from this study was that a diet rich in plant-based foods could reduce cancer incidence by as much as 40% (3). These results identified diet as a modifiable risk factor for cancer development and led to an extensive evaluation of the association between dietary patterns and cancer risk. In 2007, the World Cancer Research Fund, together with the American Institute for Cancer Research (AICR), published its Second Expert Report "Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective", an extensive systematic review addressing the causal associations between food, nutrition, physical activity and risk for various cancers. The Report stated that consumption of fruit and non-starchy vegetables “probably protects” against various cancers of the upper gastrointestinal tract, but that evidence suggesting a protective effect against other types of cancer, such as those of the lung, ovary and colon and rectum, is limited. Evidence suggesting that intake of starchy plant foods, especially when consumed whole or minimally processed, protects against colorectal cancer was found to be “probable”. The high level of dietary fiber found in these foods was thought to be responsible for their protective effect. Despite these less than compelling lines of evidence, the Expert Panel recommended consumption of a diet rich in foods of plant origin, in particular nutrient-dense foods. This recommendation was in part based on the

observation that protection against cancer is most often seen with diets that consist largely of plant-based foods. The Panel further justified their recommendation based on the observation that high intake of low energy-dense foods, such as fruits, vegetables and unrefined cereals, can protect against increased body fatness, a convincing risk factor for several types of cancer (37).

Plant-based foods are a great source of essential nutrients, such as vitamins and minerals, but also of bioactive compounds called phytochemicals. Although these non-nutrient compounds are not needed for optimal growth, development and maintenance of the human body, they are thought to be, in part, responsible for the “added health benefit” associated with consumption of a diet rich in foods of plant origin (37). Phytochemicals are classified based on their chemical structure and functional characteristics in the following categories: carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (38). Most of these compounds are secondary plant metabolites and do not play a critical role in the plant’s survival, but appear to have evolved because of their protective properties against herbivores and pathogens (39). In humans, phytochemicals may protect against multiple diseases of aging, including development of cancer via multiple mechanisms, including antioxidant activity, alteration of carcinogen metabolism via enzyme induction, increased DNA repair, arrest of cell cycle progression, promotion of apoptosis, suppression of proinflammatory responses, or inhibition of angiogenesis and metastasis (40, 41).

The nutrient and non-nutrient composition of plant-based foods can vary greatly between species, but also within a species due to genetic differences as a result of selective breeding or genetic modification (42, 43). Environmental factors, agricultural

and processing practices, storage conditions and preparation methods can also affect the composition and concentration of nutrients and non-nutrients in plant tissue, as well as their bioavailability (21, 44-49). The variation in the biological response to these compounds may be increased as a result of the differences in composition and bioavailability, and this increase in variation could contribute to the relatively weak association between consumption of plant-based foods and reduced risk for cancer, as described in the second expert report.

The goals and recommendations of the Second Expert Report were largely based on evidence derived from retrospective case-control studies and prospective cohort studies (37). Both studies rely on accurate recollection of the diet by study participants, and results may be flawed due to recall bias (50). The tendency of study participants to overreport intake of fruits, vegetables and whole grains, but underreport intake of processed foods and foods high in fat and sugar, can result in an underestimation of the association between higher intake of plant-based foods and reduced cancer risk. The use of biomarkers, such as blood and urine levels of dietary compounds and/or their metabolites, as a measure of dietary intake may be more accurate, but expensive and time-consuming methodology makes longitudinal measurements not feasible (51).

2.3. Cruciferous vegetables and cancer

Although the Expert Panel judged the evidence supporting an association between total fruit and vegetable intake and overall cancer risk to be weak (37), this conclusion does not exclude the possibility that specific groups of fruits and vegetables may have a stronger protective effect on site-specific cancers (52).

Within the different groups of non-starchy vegetables, cruciferous vegetables have gained considerable attention for their anticarcinogenic potential. Several epidemiological studies have suggested that crucifers, compared to all groups of fruits and vegetables, offer greater protection against certain types of cancer. These include cancers of the lung (5, 53), stomach (7, 53), bladder (6), colon and rectum (5, 7), prostate, and the estrogen-associated cancers of the breast (5), ovary (54), endometrium (55) and prostate (56). The protective effect of this group of vegetables is also highlighted on AICR's web site "Foods That Fight Cancer" (57); however, separate recommendations for intake of cruciferous vegetables have not been established.

Cruciferous vegetables include the edible members of the Brassica genus, named after their four-petal flowers resembling a cross. They can be divided into vegetables that form a head, such as broccoli, cauliflower, cabbage and Brussels sprouts, and so-called headless vegetables, including kale, collards and mustard. Arugula, (horse)radish and watercress, although not part of the Brassica genus, are also considered crucifers (10). Despite their vast differences in appearance, cruciferous vegetables are unique in that they all contain high levels of sulfur and nitrogen-containing glucosides called glucosinolates. These secondary metabolites, although not directly involved in growth, development and reproduction of the crucifer, are generally

believed to have evolved as part of the plant's protection against herbivory (58). Likewise, glucosinolates are not an essential component of the human diet; however, they are believed to be responsible for the greater protective effect that crucifers seem to offer compared to fruits and vegetables in general (59).

In the plant, glucosinolates are sequestered in vacuoles (60). When the plant tissue is ruptured, for instance during mastication or food preparation, glucosinolates come into contact with the endogenous plant enzyme myrosinase. This β-thioglucosidase catalyzes the hydrolysis reaction that removes the glycoside portion from the glucosinolates, resulting in the formation of glucose and an unstable aglycone intermediate. The aglycone rearranges to form different products, including isothiocyanates, nitriles, epithionitriles, oxazolidine-2-thiones, and thiocyanates (Fig. 2.4). Of these breakdown products, isothiocyanates are thought to be responsible for the biological activity of the parent glucosinolate (8, 61).

Broccoli is the second most consumed crucifer in the U.S. behind cabbage. The predominant glucosinolate hydrolysis products derived from broccoli are the isothiocyanate breakdown product indole-3-carbinol (I3C) from glucobrassicin, and the stable isothiocyanate sulforaphane (SF) from glucoraphanin (62). One of the first anticarcinogenic actions reported for broccoli-derived glucosinolate breakdown products was upregulation of the phase II detoxification enzyme NAD(P)H dehydrogenase, quinone 1 (NQO1) by SF (63). Phase II enzymes play an essential role in the cellular defense against damage by electrophiles and reactive oxygen species. Many of these enzymes catalyze conjugation reactions, which decrease the reactivity and simultaneously increase the water-solubility and excretion of potentially harmful

compounds (64). Expression of many of the phase II enzymes, including NQO1, is under control of the transcription factor nuclear factor E2-related factor 2 (Nrf2). Under normal conditions, Nrf2 is sequestered in the cytoplasma by kelch-like ECH-associated protein 1 (Keap1) and undergoes continuous ubiquitination and degradation. Under conditions of oxidative or electrophilic stress, Keap1 is inactivated, which allows *de novo* synthesized Nrf2 to translocate into the nucleus. There, it binds to the antioxidant response element of target genes, which upregulates their transcription (65, 66). *In vitro* studies have shown that SF is a potent inducer of nuclear Nrf2 accumulation (67, 68). This accumulation is thought to be the result of SF interacting with Keap1, thereby disrupting the Nrf2-Keap1 complex and releasing Nrf2 from its sequestered state, and/or disrupting the binding of Keap1 to newly synthesized Nrf2 molecules so they accumulate and pass into the nucleus (69, 70). Subsequent nuclear accumulation of Nrf2 was associated with increased expression of the antioxidant response element target gene heme oxygenase 1 (68). In Nrf2 knockout mice, both basal and SF-induced activity of NQO1 were shown to be substantially diminished compared to wild type mice (71). These data show that *in vitro* and *in vivo* upregulation of phase II detoxification enzymes by SF is, in part, mediated by Nrf2.

Indole-3-carbinol and its major acid condensation product 3,3'-diindolylmethane (DIM), which is formed in the stomach, were first identified as inducers of aryl hydrocarbon hydroxylase, or cytochrome P450 (CYP) 1A1 (72). Cytochrome P450 enzymes catalyze the majority of phase I detoxification reactions, including oxidation, reduction and hydrolysis, and both endogenous and xenobiotic compounds can act as substrates. Phase I reactions can lead to formation of highly reactive electrophilic

intermediates, as is the case with procarcinogens, such as benzo[*a*]pyrene. However, formation of an electrophilic intermediate activates phase II detoxification enzymes, which results in inactivation and elimination of the reactive compound, as described above (73, 74).

Indole-3-carbinol and its derivative DIM have been shown to induce gene transcription via activation of the aryl hydrocarbon receptor. Upon ligand binding, the aryl hydrocarbon receptor moves into the nucleus where it is thought to complex with its nuclear translocator and bind to the xenobiotic response element on target genes. Activation of the aryl hydrocarbon receptor induces transcription of CYPs and other phase I detoxification enzymes, but also of phase II enzymes containing a xenobiotic response element in their promoter region. Therefore, I3C and DIM are considered bifunctional inducers, whereas SF, which activates phase II enzymes only, is considered a monofunctional inducer (75-77).

Induction of phase I detoxification enzymes by I3C and DIM, and subsequent formation of potentially harmful compounds may seem to undermine the chemoprotective properties of cruciferous vegetables. However, multiple studies have shown that I3C and DIM can protect against formation of DNA adducts by chemical carcinogens, such as aflatoxin (78, 79), heterocyclic amines and polycyclic aromatic hydrocarbons (80, 81), and cigarette smoke (82). This protective effect may be the result of activation of phase II detoxification enzymes by I3C and DIM directly via the xenobiotic response element, instead of in response to formation of reactive intermediates. Another possibility is that I3C and DIM are relatively weak inducers of phase I detoxification enzymes compared to (pro)carcinogenic compounds, thereby

inhibiting carcinogen-induced enzyme activity through competition for binding to the aryl hydrocarbon receptor.

Protection from xenobiotic insult via modulation of hepatic phase I and II detoxification enzyme activity is considered one of the main mechanisms by which broccoli exerts its anticarcinogenic effect. Chemoprotective actions of glucosinolate hydrolysis products that target cancer cells directly have also been identified. These include: modulation of drug transporters, which are involved in expelling potentially harmful compounds from the cell (83); inhibition of cell proliferation via induction of apoptosis and cell cycle arrest; modulation of hormone receptor expression; enhanced sensitivity to chemotherapeutic drugs; epigenetic regulation and inhibition of angiogenesis and metastasis (84, 85). Many of these effects have been reported in ovarian and prostate cancer cells, as well as many other cell types. These *in vitro* studies have revealed an array of molecular targets for purified glucosinolate hydrolysis products. Indole-3-carbinol, DIM and SF were shown to arrest cell cycle progression via upregulation of the cyclin-dependent kinase inhibitor p21 and the Retinoblastoma tumor suppressor protein (86-88). It was also demonstrated that these glucosinolate hydrolysis products induced apoptosis via inactivation of the Akt cell survival pathway (88, 89) and by shifting the balance between proapoptotic (Bax, Bad) and antiapoptotic (Bcl-2, Bcl-xL) members of the Bcl-2 family of apoptotic regulator proteins (90, 91). Combinations of glucosinolate hydrolysis products with chemotherapeutic or proapoptotic agents were shown to augment cellular sensitivity (89, 90, 92, 93), which is of particular interest in the treatment of advanced, chemorefractory cancers. In recent years, SF has been identified as an inhibitor of histone deacetlyase activity (94) and a mediator of changes

in DNA methylation status (95). Epigenetic modifications can lead to alterations in gene expression patterns and are thought to play an important role in cancer development. Both DNA methylation of the CpG islands in the promoter region of a gene and histone deacetylation are associated with compaction of the DNA structure, making it inaccessible to the cell's transcription machinery and resulting in gene silencing (96, 97). Sulforaphane has been shown to inhibit histone deacetylation in prostate cancer cells, which resulted in increased expression of cell cycle regulator p21, cell cycle arrest and induction of apoptosis (86, 98). The isothiocyanate has also been shown to demethylate the promoter region of cell cycle regulator cyclin D2 and concomitantly induce gene expression in prostate cancer cells, which correlates with reduced tumor aggressiveness (99).

Overall, *in vitro* studies have given the scientific community tremendous insight into the multitude of mechanisms underlying the chemoprotective effects of glucosinolate hydrolysis products derived from broccoli. However, in many of these studies cell were exposed to much higher concentrations than those that can be achieved in the plasma through dietary means (< 5 μM). Therefore, more studies using physiologically relevant concentrations, both *in vitro* and *in vivo*, are necessary to pinpoint which of the many mechanisms identified actually contribute to the added health benefit associated with increased consumption of broccoli and other crucifers in the diet.

2.4. Estrogen metabolism

A proposed mechanism of protection against estrogen-associated cancers is alteration of estrogen metabolism (9). The liver is the primary site of estrogen metabolism (100). Estrogens are metabolized mainly by the phase I detoxification enzymes CYP3A4 to form 16α-hydroxyestrogens, which retain most of the estrogenic activity, or CYP1A to form 2-hydroxyestrogens with weak estrogenic activity. A third, minor pathway of hepatic estrogen metabolism involves formation of 4-hydroxyestrogen by CYP1B1 (16, 101) (Fig 2.5). In extrahepatic tissues, such as breast, ovary and uterus, 4-hydroxylation is the predominant pathway for estrogen metabolism, although formation of 2-hydroxyestrogens has also been observed in these tissues. Both 2- and 4-hydroxyestrogens can be conjugated to glucuronides and sulfates, but when cellular levels of conjugates are low, the estrogen metabolites can undergo redox cycling to generate free radicals and form reactive quinones. In particular, the estrogen quinone derived from 4-hydroxyestrogens is a highly reactive metabolite that can form DNA adducts leading to apurinic sites. Local, extrahepatic formation of 4-hydroxyestrogens by CYP1B1 is thought to play a major role in the development of breast and endometrial cancer (22, 102). 2-Hydroxyestrogen quinones can be reduced back to 2-hydroxyestrogens by NQO1 (103) (Fig. 2.5).

2-Hydroxyestrogens, and to a lesser extent 4-hydroxyestrogens, can also be methylated by catechol-*O*-methyltransferase (COMT) (18) (Fig. 2.5). In particular, the methylated product 2-methoxyestradiol has emerged as a promising anticancer agent and is currently being evaluated in phase I and II clinical trials. This endogenous metabolite of estradiol has been shown to selectively inhibit growth of human breast

(104, 105), ovarian (106) and cervical cancer cells (107), and suppress growth of human prostate cancer cells *in vitro* (108, 109). The inhibitory effect of 2-methoxyestradiol on tumor growth and development *in vivo* has been demonstrated in breast (110, 111) and prostate cancer models (112, 113). In humans, 2-methoxyestradiol was observed to be well tolerated after oral administration with evidence of anti-cancer activity in patients with advanced disease, but its therapeutic potential appears limited by its low oral bioavailability (19, 20, 114).

Various mechanisms that underlie the anticancer effects of 2-methoxyestradiol have been elucidated *in vitro*. Exposure to the estrogen metabolite has been shown to cause cell cycle arrest either as a result of faulty spindle formation and subsequent microtubule disruption (104, 115), or inactivation of cyclin-dependent kinase 1, which promotes cell cycle progression in its active form (104, 116). 2-Methoxyestradiol has also been shown to induce apoptosis via inactivation of the antiapoptotic regulator Bcl-2 (105, 117). Besides its direct effects on cancer cells, 2-methoxyestradiol is also thought to play a role in halting angiogenesis, the formation of new blood vessels reaching out toward a tumor in response to tumor hypoxia (118). Under hypoxic conditions, the transcription factor hypoxia-inducible factor-1 mediates secretion of vascular endothelial growth factor, a potent angiogenic agent. 2-Methoxyestradiol was found to inhibit the expression, nuclear accumulation and transcriptional activity of hypoxia-inducible factor-1, thereby blocking angiogenesis (115). Angiogenesis is essential for the growth, progression and metastasis of a solid tumor (119). Thus, therapeutic agents with antiangiogenic properties, such as 2-methoxyestradiol, are of particular interest for the treatment of tumors that have advanced to later stages in the carcinogenic process.

Compared to its parent compound 17β-estradiol, 2- methoxyestradiol has been shown to have very low affinity for the estrogen receptor (120, 121). Furthermore, the antiproliferative and proapoptotic effects of 2-methoxyestradiol were shown to be unaltered in the presence of an estrogen receptor agonist (106, 122), indicating that the anticarcinogenic actions of 2-methoxyestradiol are mediated independently from the estrogen receptor. This makes 2-methoxyestradiol an attractive candidate for the treatment of hormone-refractory cancers that no longer respond to endocrine manipulations.

Several studies have shown that the anticarcinogenic actions of 2-methoxyestradiol have little to no effect on non-tumorigenic cells (104, 106, 107, 123). This indicates that 2-methoxyestradiol targets cancer cells specifically, which may minimize the incidence of cytotoxic side-effects during treatment.

Taken together, these studies emphasize the therapeutic potential of 2-methoxyestradiol. New formulations and delivery methods are currently being tested to improve its low oral availability, but thus far results have been inconclusive. Another approach to increase blood plasma levels of 2-methoxyestradiol in a non-invasive manner is through changes in endogenous estrogen metabolism.

2.5. Glucosinolate hydrolysis products and estrogen metabolism

Indole-3-carbinol supplementation has been shown to increase the 2-hydroxy- to 16α-hydroxyestrogen ratio (10), ostensibly via induction of CYP1A1/2 (85, 124). Indole-3-carbinol treatment is also associated with reduced risk of cancer of the breast (11, 12, 125, 126), ovary (13) and prostate (14). It has been suggested that the inverse

association between formation of 2-hydoxyestrogens and risk of estrogen-associated cancers is due to loss of estrogenicity, since 2-hydroxyestrogens display much weaker estrogenic activity compared to 17β-estradiol or 16α-hydroxyestrogens. However, further oxidation of 2-hydroxyestrogens to estrogen quinones could lead to formation of stable DNA adducts, which may initiate carcinogenesis (15-17). In contrast, 2-methoxyestradiol has potent anticarcinogenic properties, as described above. The observed reduction in risk for estrogen-associated cancers with increased consumption of cruciferous vegetables may therefore relate to increased formation of 2-methoxyestradiol from 2-hydroxyestradiol. Presently, the relationship between glucosinolate hydrolysis products derived from broccoli and these estrogen metabolites is essentially unknown.

2.6. Quercetin and kaempferol

Although glucosinolate breakdown products are traditionally thought to be solely responsible for the chemoprotective effect of broccoli, other compounds present in the plant tissue may also contribute to its anticancer potential. These include the flavonoids, or more specifically the flavonols quercetin and kaempferol. Flavonols are a subclass of polyphenolic compounds that can be found in a wide variety of fruits, vegetables and grains. Flavonol-rich foods include teas, apples, berries, onions and broccoli (127, 128). A limited number of epidemiological studies have shown an inverse association between consumption of flavonoids and cancer risk (129-133). The chemoprotective effects of flavonoids, including quercetin and kaempferol, have been attributed to various mechanisms, which are discussed below.

Flavonoids have been shown to exert potent antioxidant capacity, either directly through direct trapping of free radicals, or indirectly via downregulation of oxidative enzymes, such as lipoxygenase and NADPH oxidase (134). Quercetin and kaempferol were found to be the two main flavonols in broccoli, but both compounds were present as flavonol glycosides (quercetin 3-O-sophoroside and kaempferol 3-O-sophoroside are the major glycosides). The deconjugated aglycones of quercetin and kaempferol were shown to have greater antioxidant capacity compared to their respective sophorosides (135). Similar results have been found for quercetin glycosides isolated from onions (136). In contrast, glycosylation of quercetin was not shown to affect its ability to inhibit lipid peroxidation (135). The antioxidant capacities of quercetin and kaempferol have been examined *in vitro* using chemical assays (135, 137, 138). However, it is unclear whether these properties are maintained *in vivo* due to the much lower plasma and tissue levels compared to other antioxidants (such as vitamins C and E), and the rapid metabolism of these flavonoids (139, 140).

Induction of phase I detoxification enzymes is thought to increase risk for cancer through generation of potentially mutagenic intermediates (73). Transcription of the phase I enzyme CYP1A1 is under control of the aryl hydrocarbon receptor, which, upon ligand binding, associates with the xenobiotic response element, as described above. Quercetin and kaempferol were found to compete with 2,3,7,8-tetrachlorodibenzo-p-dioxin, a known human carcinogen, for binding to the aryl hydrocarbon receptor, thereby attenuating its inducible effect on CYP1A1 expression (141, 142). Inhibition of CYP1A1 activity by quercetin was also shown to prevent benzo[*a*]pyrene-induced DNA adduct formation (143). These studies suggest that the weak agonistic actions of quercetin and

kaempferol for the aryl hydrocarbon receptor may help protect against chemical carcinogens that are strong agonists.

Flavonoids are also known inducers of phase II detoxification enzymes (127), which play an important role in inactivation and elimination of carcinogenic compounds from the body, as described above. Quercetin was shown to increase transcription and activity of UDP-glucuronosyltransferase (144, 145). Steroid hormones are known substrates for this enzyme, and increased glucuronidation, followed by rapid excretion of the polar conjugates, may therefore protect against hormone-associated cancers (146, 147). Like SF, quercetin and kaempferol have been demonstrated to increase transcription and activity of the anticarcinogenic phase II detoxification marker enzyme NQO1 (136, 148, 149). In contrast, quercetin-3-O-sophoroside was not shown to induce NQO1 activity (136).

Flavonoids and glucosinolate hydrolysis products have been shown to have overlapping targets for inhibition of cancer-related signaling pathways (134, 150). Quercetin and kaempferol were found to upregulate expression of p21 and concomitantly induce cell cycle arrest in human breast (151, 152), cervical (153) ovarian (154) and prostate cancer cells (155). Both flavonoids were also found to induce apoptosis via downregulation of the Akt survival signaling pathway (154, 156, 157) and an increase the ratio of proapoptotic to antiapoptotic regulators of the Bcl-2 family (152, 158, 159).

Multidrug resistance is a major impediment to the effective treatment of advanced cancers (160). Resistance to chemotherapeutic agents is, in part, mediated by overexpression of transmembrane transporter proteins, which results in enhanced

efflux of xenobiotics (161). Quercetin and kaempferol have been shown to inhibit transcription and/or activity of various transporters, including P-glycoprotein, multidrug resistance associated proteins and breast cancer resistance protein (162, 163). Inhibition of these transport proteins has been demonstrated to markedly increase the sensitivity of drug-resistant cancer cells to chemotherapeutics, including breast (164, 165), prostate (166, 167), ovarian (168, 169) and cervical cancer cells (170, 171). These findings suggest that SF, quercetin and kaempferol may positively interact to maximize inhibition of transport proteins involved in multidrug resistance.

2.7. Selenium

Selenium is an essential trace element that is required for various biological processes, including thyroid hormone synthesis and metabolism, immune function, and the proper functioning of a number of selenium-dependent enzymes, also known as selenoproteins. Although 35 selenoproteins have been identified, the function of only a few of these proteins has been elucidated. These include the glutathione peroxidases and thioredoxin reductases, antioxidant enzymes that are part of the overall defense of the cell against oxidative stress (172). Selenium deficiency is associated with hypothyroidism, leading to goiter, cretinism and recurrent miscarriage. Other clinical disorders that are observed with selenium deficiency are Keshan disease, an endemic cardiomyopathy that is linked to increased virulence of the normally dormant Coxsackie virus, and Kashin-Beck disease, a chronic, degenerative osteoarthropathy (172, 173). Insufficient intake of selenium is also thought to increase risk for cancer. Dietary intake of selenium and serum selenium levels have been shown to be inversely correlated with

cancer incidence and mortality (174, 175). These studies suggest that adequate or supplementary intake of selenium may have a protective effect on the development of the disease.

Selenium is thought to mediate its anticarcinogenic effect through multiple mechanism. Besides its antioxidant role as part of the glutathione peroxidases and thioredoxin reductases (172), selenium has also been shown to increase repair of oxidative stress-induced DNA damage (176). Selenium has been found to modulate molecular targets in anticancer signaling pathways similar to those affected by glucosinolate breakdown products and flavonols, as described previously. These include upregulation of p21 (177) and inactivation of Akt (178), accumulation of Bax and downregulation of Bcl-xL (178) and Bcl-2 (179). Selenium was also shown to inhibit proangiogenic factors (180) and growth of endothelial cells (181), indicating an inhibitory role in angiogenesis.

Selenium has gained much attention as a cancer preventive agent since the publication of the Nutritional Prevention of Cancer (NPC) trial. This placebo-controlled study was originally designed to determine whether supplemental selenium (as selenized yeast) would decrease the incidences of recurrent basal and squamous cell skin cancers. Daily supplementation with 200 µg selenium was not shown to protect against these skin cancers. However, secondary endpoint analyses showed that selenium supplementation reduced the incidence of total, lung, colorectal and prostate cancer (182).

The major selenium species in selenized yeast is selenomethionine (183), which, based on the outcomes of the NPC trial, was selected for the more recent Selenium and

Vitamin E Cancer Prevention Trial (SELECT). This large study, with over 35,000 participants, sought to determine whether selenium, vitamin E or both could prevent prostate cancer and other cancers in healthy men. After five years of participant follow-up, it was shown that daily supplementation of 200 µg selenomethionine (either alone or in combination with vitamin E), failed to lower the incidence of prostate cancer (184). This lack of benefit was also shown with extended participant follow-up (185). A key reason for this lack of effect may have been the choice of selenium formulation. Although selenomethionine was the primary bioactive component in selenized yeast, other forms of selenium present in the yeast, such as Se-methylselenocysteine (Se-MSC) (183) may have contributed to the observed reduction in cancer incidence. A study comparing the activity of selenized yeast to selenized garlic in prevention of chemically induced mammary carcinogenesis found that selenized garlic was more effective in suppressing the carcinogenic process, despite lower total tissue selenium accumulation compared to selenized yeast. Subsequent speciation analysis showed that the main selenium species in selenized garlic was γ-glutamyl Se-MSC, a metabolite of Se-MSC. Compared to selenized yeast, selenized garlic also contained a larger proportion of total selenium as Se-MSC (186). These results suggest that selenomethionine may not have been solely responsible for the reduction in cancer incidence observed in the NPC trial, and that Se-MSC may deserve further study.

It should also be noted that the strongest protective effect in the NPC trial was seen in people with the lowest tertile of plasma selenium at the start of the trial (187). In contrast, the subjects enrolled in the SELECT trial had higher initial plasma levels of selenium than those in the NPC trial (188). Supplementation of selenium has been

shown to increase glutathione peroxidase activity, however, this correlation was found to be weaker with increased selenium status (189). These observations suggest that an increase of selenium plasma concentrations above a certain threshold level may not result in further protection against cancer development, which could have contributed to the observed lack of effect in the SELECT trial

2.8. Crucifers and selenium

Crucifers typically contain low amounts of selenium, but have the ability to actively accumulate selenium when grown on selenium-rich soil. As a result, the level of selenium found in the plant tissue can be several orders of magnitude greater than normal (190, 191). Selenium is primarily taken up from the soil as inorganic selenate. In the plant, it is sequentially reduced to selenite and selenide before being incorporated into the amino acid selenocysteine. This organic form of selenium can be incorporated into selenoproteins in place of cysteine. Another fate of selenocysteine is conversion to the amino acid selenomethionine, which is randomly incorporated into proteins in place of methionine. In crucifers and other selenium accumulators, such as members of the allium species, selenocysteine can also be methylated to form Se-MSC (192, 193). Analysis of selenium-enriched crucifers has shown that Se-MSC is the major selenium species in these vegetables. In contrast, non-accumulators, such as wheat, when grown on high-selenium soils, were shown to incorporate selenium as selenomethionine (183).

Multiple animal studies have demonstrated the enhanced anticarcinogenic potential of selenium-enriched broccoli and other cruciferous vegetables. Selenium-enriched Japanese radish sprouts and broccoli have shown greater inhibition of

chemically induced mammary carcinogenesis in the rat compared to their low-selenium equivalents (194, 195). Selenium-enriched broccoli and broccoli sprouts have been shown to reduce the number of aberrant colon crypts in chemically induced colon cancer in the rat (195), and selenium enriched broccoli has been shown to decrease intestinal tumorigenesis in the Min mouse model of colon carcinogenesis (196) to a greater extent than their respective low-selenium equivalents. Compared to selenomethionine or inorganic selenite, Se-MSC has demonstrated greater antitumor effects in a xenograft model of human prostate cancer (197). In human prostate cancer cells, SeMSC but not selenite altered expression of various collagen genes, indicating anticancer activity through modulation of the extracellular matrix and stroma (198). These studies strongly suggest a potential role for Se-MSC, the main form of selenium in cruciferous vegetables, and/or its metabolite(s) in cancer prevention. However, more definitive studies are needed.

2.9. Hypotheses and aims

Central hypothesis:

Broccoli bioactives, as part of the whole vegetable, have greater anticarcinogenic activity compared to purified compounds due to interactive effects between multiple bioactives present in the plant tissue.

Study 1: Broccoli enhances metabolism of estradiol toward formation of 2-methoxyestradiol in vitro and ex vivo

Hypothesis:

Glucosinolate breakdown products derived from broccoli enhance formation of 2-methoxyestradiol via combined upregulation of CYP1A and NQO1 activity.

Aim 1:

Determine the effect of glucosinolate breakdown products, either derived from the whole food or as purified compounds, on CYP1A and NQO1 activity in murine Hepa-1c1c7 and chicken LMH hepatocellular carcinoma cells, or in chicken liver and ovary.

Aim 2:

Determine the effect of glucosinolate breakdown products, either derived from the whole food or as purified compounds, on formation of 2-methoxyestradiol from exogenous 17β-estradiol in Hepa-1c1c7 cells, or in chicken liver.

Study 2: Differential and interactive effects of broccoli bioactives on cancer cell growth and enzyme induction

Hypothesis:

Broccoli inhibits growth of the human prostate adenocarcinoma cell line LNCaP and the human ovarian adenocarcinoma cell line OVCAR-3 via enhanced formation of 2-methoxyestradiol, but this inhibition is not solely due to the glucosinolate breakdown products I3C and SF.

Aim 1:

Determine the effects of glucosinolate breakdown products on 17β-estradiol-induced growth of LNCaP and OVCAR-3 cells, both in the presence and absence of COMT, the enzyme that catalyzes formation of 2-methoxyestradiol (Fig. 2.5).

Aim 2:

Compare the growth-inhibitory effects of glucosinolate breakdown products in broccoli to those of purified I3C plus SF in LNCaP and OVCAR-3 cells.

Aim 3:

Evaluate the interactive effects between SF, quercetin and kaempferol on upregulation of NQO1activity in Hepa-1c1c7 cells.

Study 3: Selenium enrichment of broccoli alters its bioactivity via changes in the glucosinolate profile

Hypothesis:

Enhanced upregulation of CYP1A and NQO1 activity by selenium-enriched broccoli is due to accumulation of selenium as Se-MSC in the plant tissue.

Aim 1:

Determine the effects of low-selenium broccoli and high-selenium broccoli on CYP1A and NQO1 activity in Hepa-1c1c7 cells

Aim 2:

Determine the effect of low-selenium broccoli on CYP1A and NQO1 activity in Hepa-1c1c7 cells in combination with selenium supplementation (as selenite or Se-MSC).

Aim 3:

Determine the effect of neoglucobrassicin hydrolysis products on enzyme activity in Hepa-1c1c7 cells.

2.10. Rationale and experimental approach

The endogenous estrogen metabolite 2-methoxyestradiol has shown promising anticarcinogenic potential in multiple *in vitro* and *in vivo* studies, but its effectiveness in clinical trials is greatly hampered by its low oral bioavailability. New formulations of 2-methoxyestradiol with presumed improved bioavailability are currently under investigation (199). An alternate approach is to increase endogenous levels of 2-methoxyestradiol through changes in estrogen metabolism, which may also lower circulating levels of the parent compound 17β-estradiol and concomitantly reduce its mitogenic effects. Glucosinolate breakdown products derived from broccoli have been shown to alter estrogen metabolism toward formation of 2-hydroxyestrogens, the precursors to 2-methoxyestrogens (10, 85, 124). However, the impact of glucosinolate breakdown products on formation of endogenous 2-methoxyestradiol is unknown.

The *in vitro* models chosen to fill this gap in the literature were the mouse hepatoma cell line Hepa-1c1c7 and the chicken hepatoma LMH cells. Hepa-1c1c7 cells have been used extensively in drug metabolism research and are a well-established, robust tool for determining the effect of xenobiotics on hepatic detoxification enzymes, including CYP1A and NQO1. LMH cells were used to determine the impact of glucosinolate breakdown products on chicken hepatic detoxification enzyme activity. Not only have detoxification enzymes not been studied previously in this model, but data from this model can be expected to most readily compare to studies in the whole chicken. The egg-laying hen (*Gallus domesticus*) was chosen for *in vivo* studies, since this is the only animal model of spontaneous ovarian cancer, with pathogenesis similar

to the human disease (200). The outcomes of this study may serve as a rationale for dietary intervention studies to determine the impact of glucosinolate breakdown products on the development of ovarian cancer in the chicken.

The growth inhibitory effects of glucosinolate breakdown products via alteration of estrogen metabolism were also examined in the human prostate adenocarcinoma cell line LNCaP and the human ovarian adenocarcinoma cell line OVCAR-3. Both cell lines have been shown to be estrogen-responsive (13, 201) and, individually, purified I3C and SF have been shown to inhibit their growth (86, 93, 202, 203). It is currently unknown whether this inhibitory effect is, in part due to enhanced formation of 2-methoxyestradiol by glucosinolate breakdown products.

Broccoli contains numerous bioactive compounds, which may interact if the whole food is ingested. Possible interactive effects on enzyme activity (Hepa-1c1c7 cells) and cancer cell growth (LNCaP and OVCAR-3 cells) were determined by comparing the effect of a broccoli extract to that of the purified compounds I3C, SF, quercetin and kaempferol.

Broccoli is an active accumulator of selenium, and selenium enrichment has been shown to enhance its anticarcinogenic properties, including enhanced activity of detoxification enzymes (21, 193). Increased uptake of selenium by the plant has been found to alter its glucosinolate profile, which may impact enzyme activity (21, 204). Whether the observed enhancement of enzyme activities is due to accumulation of selenium in the plant tissue or to increased levels of glucosinolates is currently not known. Furthermore, it is not clear whether the selenium species stored in broccoli (Se-MSC) is more active than inorganic forms. Therefore, the effect of glucosinolate

hydrolysis products and selenium, either as purified compounds or as part of the whole plant extract, on NQO1 activity was examined in Hepa-1c1c7 cells.

2.11. Figures

Figure 2.1. Global cancer incidence. Estimated age-standardized incidence rate per 100,000. All cancers excluding non-melanoma skin cancer: both sexes, all ages (205).

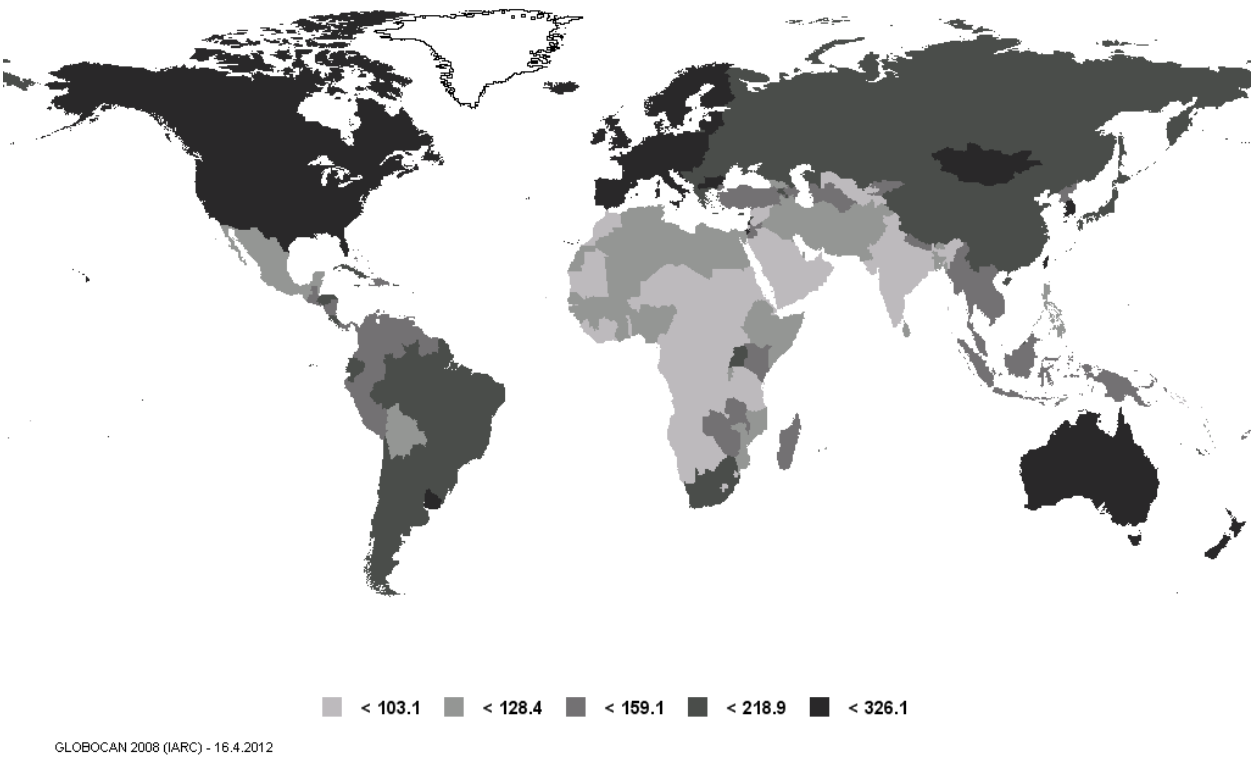


Figure 2.2. Global incidences of female estrogen-associated cancers. Estimated age-standardized incidence rate per 100,000. Breast, cervix uteri, corpus uteri, ovary, all ages (205).

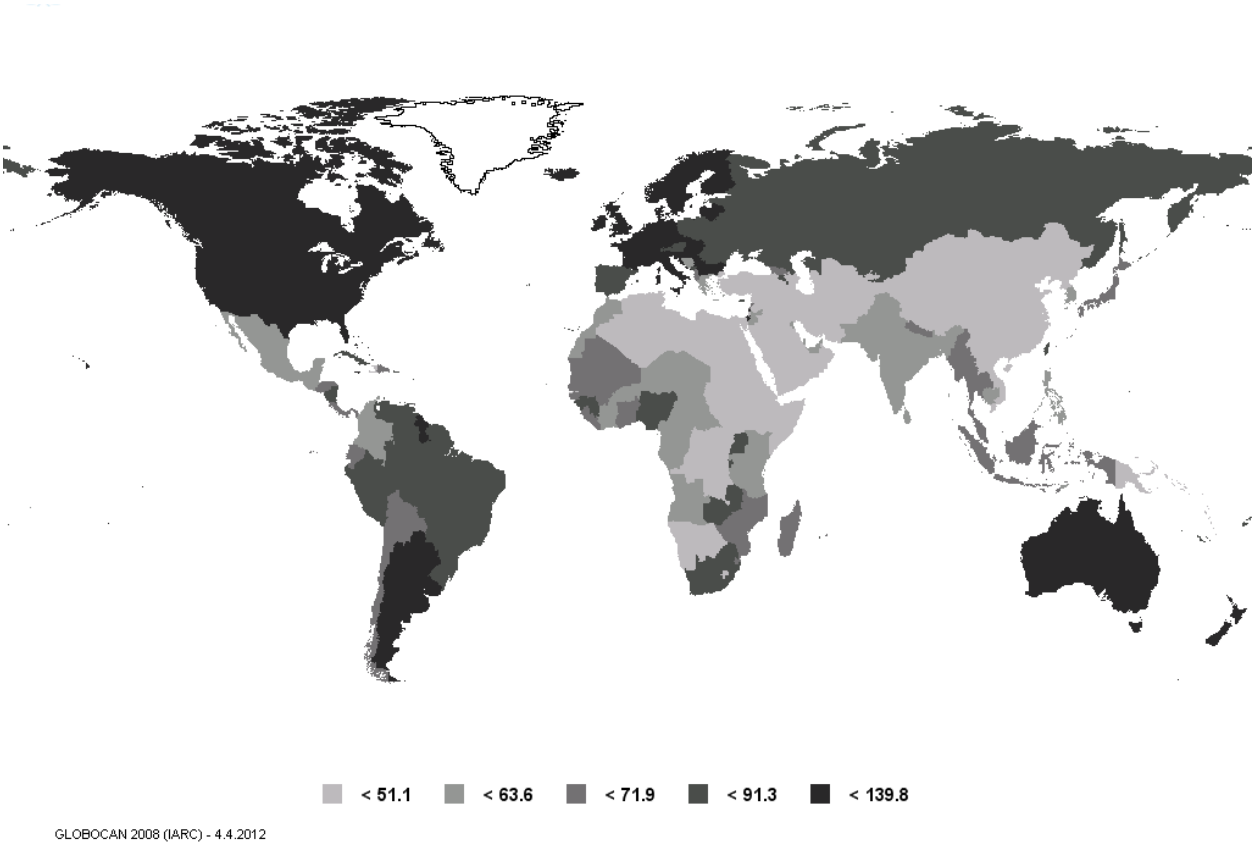


Figure 2.3. Global incidence of prostate cancer. Estimated age-standardized incidence rate per 100,000. Prostate, all ages (205).

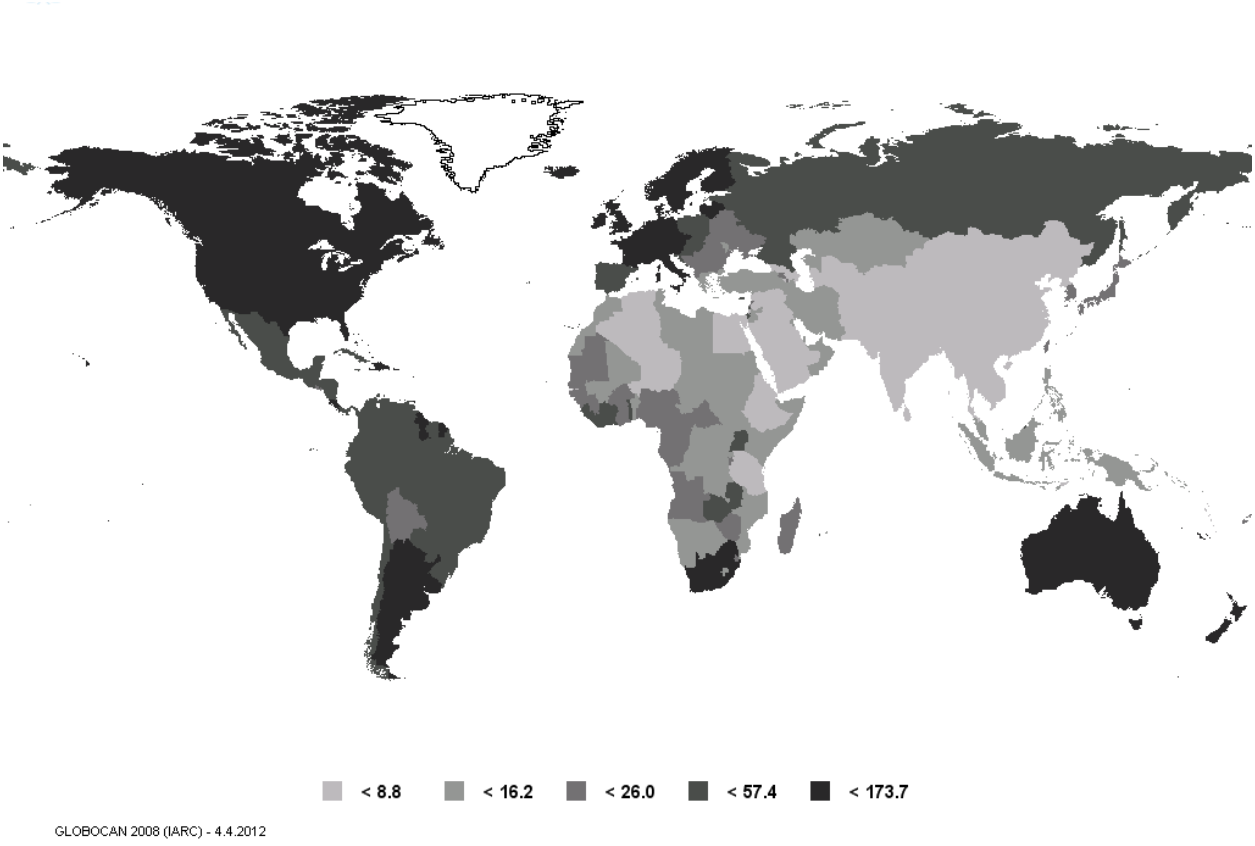


Figure 2.4. Glucosinolate hydrolysis. Myrosinase-catalyzed hydrolysis leads to formation of glucose and an unstable aglycone, which rearranges to form isothiocyanates, nitriles, epithionitriles, oxazolidine-2-thiones and thiocyanates, depending on the specific glucosinolate. Formation of nitriles is favored in the presence of epithiospecifier protein (ESP). Brackets indicate unstable intermediates; R: variable side chain. Reproduced, with permission of Annual Reviews, from Halkier BA, Gershenzon J. Biology and biochemistry of glucosinolates. Annu Rev Plant Biol. 2006;57:303-33.

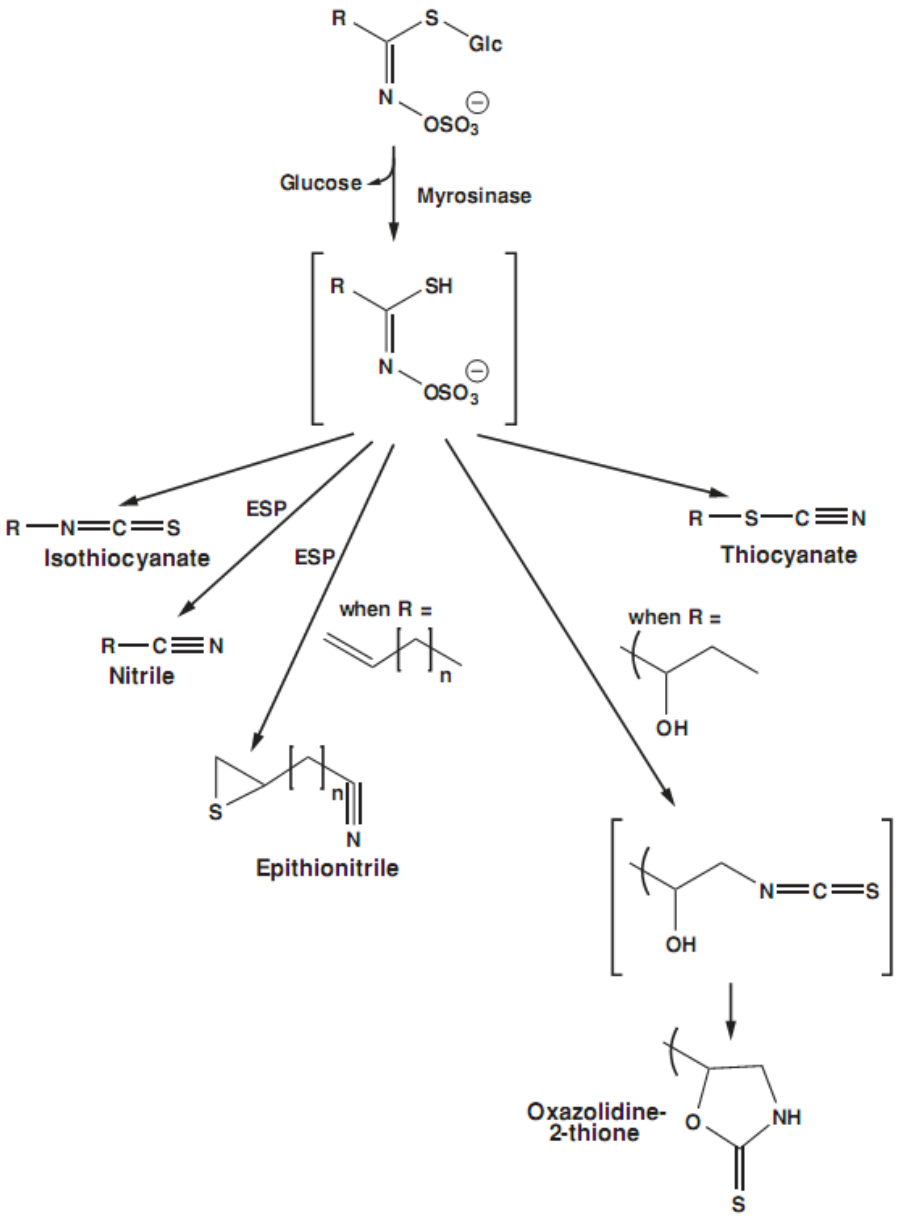
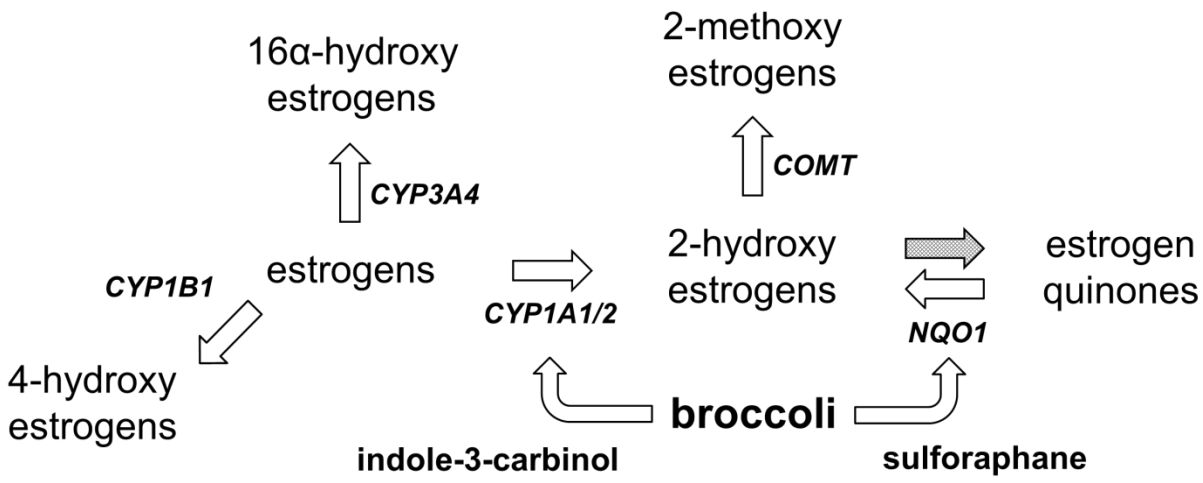


Figure 2.5. Estrogen metabolism. Estrogens are hydroxylated at the C-2, C-4 or C-16 position by cytochrome P450 (CYP) 1A1/2, 1B1 or 3A4, respectively. 2-Hydroxy-estrogens are subsequently methylated by catechol-*O*-methyltransferase (COMT) to form 2-methoxyestrogens, or form quinones, which can be reduced back to 2-hydroxyestrogens by NAD(P)H dehydrogenase, quinone 1 (NQO1).



CHAPTER 3

Broccoli enhances metabolism of estradiol toward formation of 2-methoxyestradiol *in vitro* and *ex vivo*

3.1. Introduction

Consumption of cruciferous vegetables, such as broccoli, cauliflower and cabbage, is associated with reduced risk for various cancers, including prostate cancer (56, 206-208) and the estrogen-associated cancers of the breast (209-211), ovary (54, 212) and endometrium (55). The chemoprotective effect of crucifers is mainly attributed to high levels of glucosinolates and their bioactive breakdown products, the isothiocyanates (8). The predominant glucosinolate hydrolysis products derived from broccoli are sulforaphane (SF) and indole-3-carbinol (I3C) (62).These hydrolysis breakdown products are thought to exert their anticarcinogenic effects, in part, via increased activity of phase I and II detoxification enzymes, such as cytochrome P450 1A (CYP1A) and NAD(P)H dehydrogenase, quinone 1 (NQO1) (63, 72).

A proposed mechanism of protection against estrogen-associated cancers is alteration of estrogen metabolism (9). Estrogens are metabolized mainly in the liver via multiple pathways, as shown in Figure 2.5. Increased formation of the weakly estrogenic 2-hydroxygens, presumably via increased CYP1A activity (85, 124), is associated with reduced risk of cancer of the breast (11, 12, 125, 126), ovary (13) and prostate (14). However, accumulation of 2-hydroxyestrogens may lead to formation of DNA adducts (15-17).

2-Hydroxyestrogens can also be methylated to 2-methoxyestrogens by catechol-*O*-methyltransferase (COMT) (18) (Fig. 2.5). In particular, 2-methoxyestradiol has shown promising anticarcinogenic effects in multiple cancer cell lines as well as animal models, and its therapeutic potential as an anti-cancer drug is currently evaluated in clinical trials (199, 213).

We hypothesized that a key protective effect of crucifers against cancers influenced by estrogen status may relate not only to endogenous formation of non-estrogenic 2-hydroxy estradiol, but to anticarcinogenic 2-methoxyestrogens. Presently, the relationship between glucosinolate breakdown products and these estrogen metabolites is largely unknown. Therefore, the hypothesis was expanded to include that glucosinolate breakdown products found in broccoli redirect estrogen metabolism in favor of 2-methoxyestradiol, via enhanced formation of 2-hydroxyestradiol, as a result of CYP1A1/2 and NQO1 enzyme induction (Fig. 2.5).

The current study examines the effect of glucosinolate breakdown products found in broccoli on formation of 2-methoxyestradiol *in vitro* and *ex vivo*. *In vitro* studies were performed using the murine Hepa-1c1c7 and chicken LMH hepatocellular carcinoma cells. The egg-laying hen (*Gallus domesticus*) was chosen for *in vivo* studies. The impact of glucosinolate breakdown products on estrogen metabolism has not been determined in this preclinical model of ovarian cancer.

3.2. Methods

Plant material, chemicals and reagents. Broccoli florets (*Brassica oleracea* var. Monaco), freeze-dried and ground to a powder, were obtained from FutureCeuticals (Momence, IL) as described previously (21). Unless noted otherwise, chemicals and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Cell culture. Murine Hepa-1c1c7 and chicken LMH hepatocellular carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Hepa-1c1c7 cells were maintained in Eagle's minimum essential medium with alpha modification, supplemented with 2.2 g/L sodium bicarbonate and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). LMH cells were grown in culture vessels pre-coated with 0.1% gelatin and maintained in Waymouth's MB 752/1 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

In vitro treatments. Indole-3-carbinol, SF, DIM (LKT Laboratories, Inc. St. Paul, MN), α-naphthoflavone (CYP1A inhibitor, CYP1A-i), β-naphthoflavone (β-NF) and Ro 41-0960 {{}}(COMT inhibitor, COMT-i) were dissolved in DMSO, sterile-filtered and stored at -20°C. Dicumarol (NQO1 inhibitor, NQO1-i) was dissolved in 1 N NaOH, further diluted in 50 mM sodium phosphate buffer, pH 7.4, sterile-filtered and stored at 4°C. Broccoli powder hydrolysis to release I3C and SF from their respective parent glucosinolates was performed as described previously (21). In brief, broccoli (*Brassica oleracea* var. Majestic) was harvested, cut 2 inches below the floret and freeze-dried. Freeze-dried broccoli powder (50 mg/mL) was suspended in water for 24 h at room temperature away from light to allow for maximum formation of glucosinolate hydrolysis

products. Broccoli hydrolysate was sterile-filtered to produce an aqueous extract prior to treatment of cells. The hydrolyzed broccoli extract was added to the culture medium at 1-10% final concentration, equivalent to 0.5-5 mg freeze-dried broccoli powder per mL culture medium; all other treatments were added to the culture medium at 0.1% final concentration.

Sulforaphane and I3C analysis. Sulforaphane and I3C were extracted from the hydrolyzed broccoli extract and analyzed by reverse-phase HPLC with UV detection as described previously (21).

Animal study and experimental design. The animal protocol was approved by the University of Illinois Animal Care and Use Committee. Eighteen single-comb White Leghorn hens (*Gallus domesticus*) between 6-12 months of age were obtained from the Poultry Research Facility, University of Illinois at Urbana-Champaign. Hens were individually housed in wire-bottom cages, provided with feed and water ad libitum and exposed to a photoperiod of 16 h light/8 h dark, with lights on at 05:30 h and lights off at 21:30 h. Hens were provided with standard chicken feed and acclimated for 3 days. On day 3, animals were randomly assigned to 3 experimental groups (n = 6). Experimental diets included standard feed (control), 10% broccoli powder (broccoli), or purified I3C (2,500 ppm). Animals were fed experimental diets for 7 days. Body weights were measured before and after the 7-day feeding trial.

Tissue collection. Blood was collected from the brachial vein before and after the 7-day feeding trial. Blood was stored at 4°C for 24 h and centrifuged for 30 min at 2,000 x *g*. Serum was collected and stored at -80°C until further analysis. Animals were euthanized by CO_2 asphyxiation, and livers and ovaries were collected. Post-ovulatory

follicles were removed from ovary; tissues were weighed, snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Enzyme activity. For measuring enzyme activity *in vitro*, Hepa-1c1c7 cells were seeded at 1 x 10^4 cells per well into flat bottom 96-well plates (Corning); LMH cells were seeded at 1.5 x 10^6 cells per 100 mm culture dish (Corning). Cells were allowed to adhere overnight, and medium was replaced with medium containing treatments. Hepa-1c1c7 cells were treated for 24 h with hydrolyzed broccoli extract at a final concentration of 2.5% (equivalent to 1.25 mg freeze-dried broccoli powder per mL culture medium), 0.125 μM I3C plus 1.25 μM SF, or 1 μM β-NF in the absence or presence of 10 μM CYP1A-i or 50 μM NQO1-i. LMH cells were treated for 48 h with 2.5% hydrolyzed broccoli extract, 2.5-50 μM I3C or 0.25-12.5 μM DIM. After 24 or 48 h incubation (to ensure upregulation of enzymes), medium was removed from Hepa-1c1c7 or LMH cells, respectively, and cells were washed with phosphate-buffered saline, pH 7.4. Hepa-1c1c7 cells were permeabilized by adding 50 μL filtered saturated digitonin per well and cells were incubated at 37°C for 10 min, followed by horizontal agitation at 100 rpm for 10 min. LMH cells were trypsinized, washed with PBS, lysed in 0.25 mL 0.05% deoxycholate solution and stored at -80°C until further analysis. Fifty μL cell lysate per well was used to measure CYP1A or NQO1 activity.

For measuring enzyme activity *in vivo*, microsomal and cytosolic fractions were prepared from liver and ovary as described previously (21). Fractions were stored at -80°C until further analysis. Fifty μL microsomal or cytosolic fraction per well was used to measure CYP1A or NQO1 activity, respectively.

The activity of CYP1A was measured as ethoxyresorufin *O*-deethylase activity (214) in black-walled, flat bottom 96-well plates (Corning, Corning NY), with modifications for measurement in whole cells and use of a plate reader. One hundred µL incubation mixture containing glucose-6-phosphate dehydrogenase at 1 unit/well, 8 µM ethoxyresorufin, 50 µM dicumarol and 8 µM $MgCl_2$ in 0.05 M Tris-HCl, pH 7.4, was added per well. After 5 min incubation at 37°C, 100 µL initiation mixture containing 5 mM glucose-6-phosphate and 0.5 mM nicotinamide adenine dinucleotide phosphate reduced NADPH in 0.05 M Tris-HCl, pH 7.4, was added per well to start the *O*-deethylation of ethoxyresorufin. Following a 5 min incubation period at 37°C, the formation of resorufin was measured fluorometrically using a microplate fluorescence reader (model FL_X800, BioTek, Winooski, VT) at excitation and emission wavelengths of 528 nm and 590 nm, respectively, at 37°C for 30 min. Amounts of resorufin formed during the linear part of the reaction were calculated using a resorufin standard curve and specific activity was expressed as pmol resorufin formed/min/mg protein.

The activity of NQO1 was measured in clear, flat bottom 96-well plates according to the method of Prochaska and Santamaria (215) with modification (216). The reaction was followed for 5 min using a microplate spectrophotometer (model MicroQuant, BioTek). Specific activity was expressed as nmol MTT reduced/min/mg protein.

Protein content was measured in clear, flat bottom 96-well plates using the Bio-Rad protein assay (217) with bovine serum albumin standards as per manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Absorbance was measured using a microplate spectrophotometer (model MicroQuant, BioTek).

Formation of 2-methoxyestradiol in Hepa-1c1c7 cells. Cells were plated at 1.5 x 10^6 cells per 100 mm culture dish (Corning) and allowed to adhere overnight. Medium was replaced with medium containing 10% estrogen-depleted fetal bovine serum (Cell Media Facility, University of Illinois at Urbana-Champaign). Cells were then exposed to hydrolyzed broccoli extract at 2.5% final concentration or 1 µM β-NF for 24 h (to ensure upregulation of enzymes). Culture medium was replaced, followed by 24 h simultaneous exposure to 10 µM 17β-estradiol plus hydrolyzed broccoli extract at 2.5% final concentration, or 1 µM β-NF in the absence or presence of 10 µM CYP1A-i, 50 µM NQO1-i or 3 µM COMT-i.

Formation of 2-methoxyestradiol in liver S9 fractions. Liver S9 fractions were prepared as described previously (21). Fractions containing 1 mg/mL protein were incubated with 10 µ M 17β-estradiol, 1.5 mM NADPH, 5 mM $MgCl_2$ and 20 mM ascorbic acid in 0.05 M Tris-HCl, pH 7.4, at 37°C for 30 min.

Extraction of 2-methoxyestradiol. Collected culture medium, serum or liver S9 fractions were filtered through a 0.45 µm filter (Corning) and the pH was adjusted to 5.0 by adding 2 M sodium acetate, pH 5.0. β-Glucuronidase type H-1 from Helix pomatia was added at approximately 1,500 units/mL sample, and the samples were incubated at 37°C for 18 h. Methanol was added to each sample to a final concentration of 10%, and 2-fluoroestradiol was added as an internal standard to each sample at a final concentration of 1 µg/mL. Each sample was applied to an Oasis HLB solid phase extraction cartridge (Waters Co., Milford, MA), conditioned with 3 mL methanol and 3 mL distilled water. Cartridges were washed with 3 mL each of 5% methanol and 2% ammonium hydroxide in distilled water, 3 mL 40% methanol and 2% ammonium

hydroxide in distilled water, and 3 mL distilled water. Samples were eluted with 3 mL 10% methanol in ethanol, and samples were evaporated under a continuous stream of nitrogen. Samples were dissolved in 50% methanol in distilled water containing 2 mM ascorbic acid. Internal standard and 2-methoxyestradiol were added to blank matrix samples to generate a standard curve. Samples were filtered through a 0.22 µm filter (Corning) and analyzed by high-performance liquid chromatography followed by mass spectrometry (HPLC-MS/MS), as described below. Protein content was measured as described above.

HPLC-MS/MS analysis. HPLC-MS/MS analysis was performed using an Agilent 1200 series HPLC system (Agilent Technologies, Foster City, CA), coupled with a 5500 QTRAP® mass spectrometer equipped with a positive atmospheric pressure chemical ionization (APCI) source (AB SCIEX, Foster City, CA). Ten µL of each sample or standard was injected onto a Zorbax Eclipse XDB C18 column (50 mm x 2.1 mm i.d., particle size 3.5 µm) preceded by a guard cartridge (12.5 mm x 4.6 mm i.d., particle size 5 µm; Agilent Technologies). The mobile phase, operating at a flow rate of 0.3 mL/min, consisted of a starting solvent of 40% methanol in water, maintained for 2 min, then raised to 100% methanol over 4 min, and maintained at 100% for 4 min. The mobile phase was returned to initial conditions and maintained for 5 min to re-equilibrate the column. Each solvent contained 0.1% v/v formic acid. The APCI-MS/MS conditions were as follows: spray voltage, 5500 V; drying gas, nitrogen (7.00 L/min); drying temperature, 350°C; nebulizer gas, nitrogen (60 psi); ionization source temperature, 600°C. For 2-methoxyestradiol, the intensity of fragment m/z 189.1 from the parent ion 303.2 was monitored. The parent ion of 2-fluoroestradiol could not be detected; instead,

the intensity of fragment m/z 177.0 from 273.1 (unique to 2-fluoroestradiol) was monitored. Standards in blank matrices were used to identify and quantify 2-methoxyestradiol. HPLC-MS/MS was performed by Dr. Zhong (Lucas) Li at the Metabolomics Center, University of Illinois at Urbana-Champaign.

Statistical analysis. Data were evaluated by one-way analysis of variance using SAS Statistical software (SAS Institute, Cary, NC), and values were considered to be different among treatments at $p < 0.05$ using Fisher's least significant difference procedure.

3.3. Results

Effect of glucosinolate breakdown products on enzyme activity in vitro. The effect of glucosinolate breakdown products, either as an aqueous hydrolyzed broccoli extract or as purified compounds, on CYP1A and NQO1 activity was determined in murine Hepa-1c1c7 and chicken LMH hepatoma cells. Exposure of Hepa-1c1c7 cells to hydrolyzed broccoli extract at concentrations at 1-10% in the medium resulted in a dose-dependent upregulation of CYP1A and NQO1 activity (Fig. 3.1A, B). However, loss of total cellular protein, an indicator of cellular toxicity, was observed at hydrolyzed broccoli extract concentrations greater than 2.5% (data not shown). To compare the effect of the hydrolyzed broccoli extract on detoxification enzyme activity to the effect of the individual compounds present in the extract, Hepa-1c1c7 cells were exposed to purified I3C plus SF at concentrations equimolar to that in the hydrolyzed broccoli extract, as determined previously (21). At 2.5%, the hydrolyzed broccoli extract upregulated CYP1A and NQO1 activity in Hepa-1c1c7 cells 17.0- and 3.6-fold,

respectively, compared to control. In contrast, purified I3C plus SF (I3C+SF) at 0.125 and 1.25 μM, respectively, upregulated CYP1A activity only 4.3-fold. However, the 3.4-fold increase in NQO1 activity was comparable to that obtained with exposure to the hydrolyzed broccoli extract (Fig. 3.1C, D).

In LMH cells, hydrolyzed broccoli extract at 2.5% modestly upregulated CYP1A 1.4-fold compared to control, but had no effect on NQO1 activity (Fig. 3.2A, B). Under acidic aqueous conditions, such as in the stomach, I3C forms condensation products, including DIM (218). Therefore, the effect of I3C and DIM on detoxification enzyme activity was determined in LMH cells. Purified I3C (2.5-50 μM) or DIM (0.25-12.5 μM) increased CYP1A activity in a dose-dependent manner (Fig. 3.2C), but neither compound had an effect on NQO1 activity (data not shown).

In Hepa-1c1c7 cells, background levels of CYP1A and NQO1 activity were too low to measure the impact of specific enzyme inhibitors. Thus, the effects of CYP1A inhibitor α-NF (CYP1A-i) and NQO1 inhibitor dicumarol (NQO1-i) were evaluated in the presence of β-NF, a potent bifunctional enzyme inducer. At 1 μM, β-NF increased CYP1A activity nearly 60-fold compared to control; co-treatment with 10 μM CYP1A-i decreased enzyme activity by 59% (Fig. 3.3A). Activity of NQO1 was increased 6.8-fold by β-NF compared to control, and this activity was decreased by 28% in the presence of 50 μM NQO1-i (Fig. 3.3B).

Formation of 2-methoxyestradiol in vitro. Formation of 2-methoxyestradiol from exogenous 17β-estradiol was assessed in Hepa-1c1c7 cells. Cells were pre-treated with 2.5% hydrolyzed broccoli extract for 24 h to allow for upregulation of CYP1A and NQO1 activity, followed by simultaneous exposure to hydrolyzed broccoli extract and 10 μM

17β-estradiol for 24 h. Hydrolyzed broccoli extract enhanced formation of 2-methoxyestradiol in culture medium 1.7-fold compared to untreated cells (Fig. 3.4A). To examine the effect of enzyme inhibition on formation of 2-methoxyestradiol, cells were treated with 1 μM β-NF alone or in combination with 10 μM CYP1A-i, 50 μM NQO-i or 3 μM COMT-i (Ro 41-0960, a specific inhibitor of COMT), since COMT catalyzes the methylation of 2-hydroxyestrogens to 2-methoxyestrogens. Exposure to β-NF enhanced formation of 2-methoxyestradiol 6.0-fold compared to control. Co-treatment with either the CYP1A-i or the NQO1-i decreased formation of 2-methoxyestradiol by 59% and 27%, respectively, compared to treatment with β-NF alone (Fig. 3.4B, C). Formation of 2-methoxyestradiol by β-NF was abolished in the presence of the COMT-i (Fig. 3.4D).

None of the treatments used *in vitro* affected the pH of the cell culture medium or total cellular protein levels (data not shown).

Animal diet and body weight. Due to the natural feeding behavior of the chicken, food intake could not be recorded. No differences in body, liver or ovary weight were observed between the dietary groups after the 7-day feeding trial (data not shown).

Enzyme activity in vivo. The impact of dietary broccoli bioactives on tissue detoxification enzyme activity in the chicken is unknown. Figure 3.5 shows that I3C at 2,500 ppm in the diet enhanced CYP1A activity 10-fold in liver (A) and ovary (C) compared to control diet, whereas supplementation of the diet with 10% broccoli powder did not increase enzyme activity in either tissue (B). Neither I3C nor broccoli enhanced NQO1 activity in either tissue (Fig. 3.5B, D).

Formation of 2-methoxyestradiol ex vivo. 2-Methoxyestradiol could not be detected in the serum of chickens fed the I3C-supplemented diet (data not shown),

therefore, formation of 2-methoxyestradiol from exogenous 17β-estradiol was evaluated in chicken liver S9 fractions. Compared to control, formation of 2-methoxyestradiol was increased 6-fold in liver S9 fractions from I3C-supplemented chickens (Fig. 3.6). Since broccoli supplementation to chickens did not increase CYP1A or NQO1 activity in the liver (Fig. 3.5A, B), formation of 2-methoxyestradiol was not evaluated in liver S9 fractions from these chickens.

3.4. Discussion

This study shows for the first time that broccoli bioactives alter estrogen metabolism toward formation of the anticarcinogenic metabolite 2-methoxyestradiol. In this study, it was shown that broccoli bioactives upregulated CYP1A and NQO1 activity, and increased formation of 2-methoxyestradiol from exogenous 17β-estradiol *in vitro* using the murine Hepa-1c1c7 hepatoma cell line (Fig. 3.1; Fig. 3.4A). Exposure to the potent bifunctional enzyme inducer β-NF further enhanced enzyme activity and formation of 2-methoxyestradiol (Fig. 3.3; Fig. 3.4B-D). Furthermore, enzyme inhibition resulted in a proportional decrease in 2-methoxyestradiol formation (Fig. 3.4B-D). In the chicken, I3C supplementation caused upregulation of hepatic CYP1A activity and a concomitant increase in 2-methoxyestradiol formation from exogenous 17β-estradiol by liver S9 fractions (Fig. 3.5A; Fig. 3.6). Together, these results establish proof of principle that broccoli bioactives enhance estrogen metabolism toward formation of anticarcinogenic 2-methoxyestradiol through upregulation of CYP1A and NQO1 activity.

In Hepa-1c1c7 cells, the increase in NQO1 activity was similar for purified I3C plus SF and the hydrolyzed broccoli extract. In contrast, hydrolyzed broccoli extract

upregulated CYP1A activity to a greater extent than the purified compounds. This may indicate that the amount of SF present in the hydrolyzed broccoli extract accounts for the increase in NQO1 activity, whereas additional compounds in the extract, alone or in combination with I3C, are responsible for the enhanced induction of CYP1A. Potential candidates are other hydrolysis breakdown products of indole glucosinolates, such as *N*-methoxyindole-3-carbinol (NI3C) derived from neoglucobrassicin (219). This metabolite was found to be a more efficient inducer of CYP1A compared to I3C *in vitro* (220). Although the hydrolyzed broccoli extract was not analyzed for NI3C content, a considerable amount of neoglucobrassicin was identified in the broccoli powder (21).

This study demonstrated for the first time the effect of broccoli bioactives on chicken detoxification enzyme activity *in vitro* and *in vivo*. Hydrolyzed broccoli extract, purified I3C and purified DIM upregulated CYP1A activity in chicken LMH hepatoma cells, but both the relative increase over control and the absolute enzyme activity were much lower than in the murine Hepa-1c1c7 hepatoma cells (Fig. 3.1; Fig. 3.2). Similar to these results, Uno *et al*. have shown that dioxin-induced CYP1A activity was much greater in Hepa-1c1c7 cells compared to human HepG2 hepatoma cells (221). Therefore, the well-established Hepa-1c1c7 cell line, with its low background levels and high inducibility of enzyme activity, remains the preferred model for *in vitro* characterization of detoxification enzyme modulators.

To examine the effect of broccoli bioactives on chicken detoxification enzymes *in vivo*, chickens were fed a diet supplemented with 10% broccoli powder or purified I3C at 2,500 ppm. This dose of I3C has previously been shown to increase the cytochrome P450 content of hepatic microsomes, as well as the urinary 2-hydroxy- to 16α-

hydroxyestrogen ratio in mice (222). Likewise, in this study I3C supplementation increased hepatic and ovarian CYP1A activity (Fig. 3.5A, C). Although the pattern of enzyme induction was similar between both organs, absolute enzyme activity was approximately 10-fold higher in the liver (data not shown). This is not surprising, since the liver is the main site for (xenobiotic) metabolism in the body. Broccoli supplementation did not affect CYP1A activity in the chicken liver or ovary (Fig. 3.5). These differential results between broccoli and I3C supplementation are most likely due to the much lower levels of I3C present in the broccoli powder: broccoli powder-supplementation resulted in 0.01 µmol I3C per g food (upon hydrolysis), whereas the I3C-supplemented diet contained 17 µmol I3C per g food.

Dietary broccoli had no effect on enzyme activity *in vivo*, but hydrolyzed broccoli extract was able to increase CYP1A activity in chicken hepatoma cells (Fig. 3.2A). Whereas the chicken hepatoma cells were exposed to a bolus dose of glucosinolate hydrolysis products, the intake of broccoli powder by the chicken occurred gradually throughout the day. Duration of glucosinolate hydrolysis is much shorter in the broccoli-fed chickens compared to the 24 h hydrolysis of broccoli powder at the bench prior to cell treatment, since the low pH of the stomach content most likely causes degradation and inactivation of myrosinase. Thus, the concentration of I3C (and its acid condensation product DIM) in the plasma of broccoli-fed chickens was apparently not sufficient for upregulation of CYP1A activity in the liver or the ovary.

In a 7-day feeding trial, standard freeze-dried, powdered broccoli caused no increase in chicken hepatic CYP1A activity. In contrast, indole glucosinolate-enriched and selenium-enriched broccoli increased hepatic CYP1A activity 1.4- and 1.8-fold,

respectively, compared to control (21). The effect of bioactives-enriched broccoli on chicken detoxification enzyme activity remains to be determined.

2-Methoxyestradiol, a naturally occurring metabolite of 17β-estradiol, has shown promising activity against multiple estrogen-associated cancers *in vitro*, in animal models and in clinical trials. However, its therapeutic application may be hampered due to its low oral bioavailability (19, 20). Reformulations of 2-methoxyestradiol to enhance its water solubility and bioavailability are currently being investigated in phase II clinical trials, but have thus far shown differential anticancer activity (223-227). Increasing endogenous levels of 2-methoxyestradiol may bypass its low oral bioavailability. In this study, broccoli bioactives increased formation of 2-methoxyestradiol via upregulation of CYP1A and NQO1. The impact of broccoli bioactives on formation of 16α-hydroxyestrogens was not determined. Supplementation of I3C has been shown to increase the 2-hydroxy- to 16α-hydroxyestrogen ratio. This increase was reported to be due to enhanced formation of 2-hydroxyestrogens, whereas levels of 16α-hydroxyestrogens did not change (222, 228). Therefore, enhanced activity of CYP1A and NQO1 *in vivo* may result not only in increased levels of the anticarcinogenic metabolite, but also in decreased circulating levels of the mitogenic parent compound, 17β-estradiol.

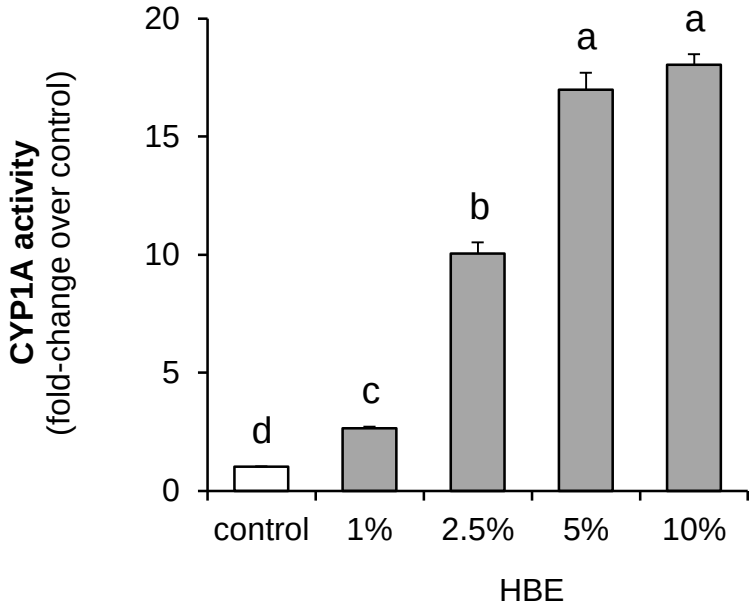
The egg-laying hen is the only model of spontaneous ovarian cancer that replicates the human disease (200). This model can be used to conduct large-scale dietary intervention studies with greater statistical power than rodent studies (229). Although in this study dietary broccoli supplementation did not increase CYP1A or NQO1 activity in the chicken, long-term feeding studies aimed at the impact of broccoli

bioactives on enzyme activity, 2-methoxyestradiol formation and development of ovarian cancer deserve further exploration.

3.5. Figures

Figure 3.1. The effect of glucosinolate breakdown products on enzyme activity in murine Hepa-1c1c7 cells. Cells were exposed to hydrolyzed broccoli extract (HBE, 1-10%) or I3C (0.125 µM) plus SF (1.25 µM) (I3C+SF). Enzyme activity was measured after 24 h exposure time. Data, expressed as fold-change over control, represent mean ± SEM; n=4. Values that differ are indicated by different letters ($p < 0.05$).

A.



B.

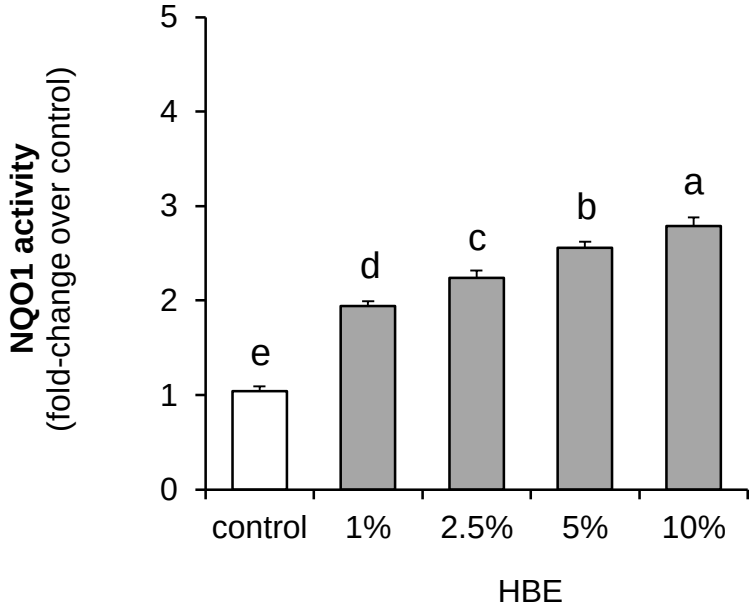
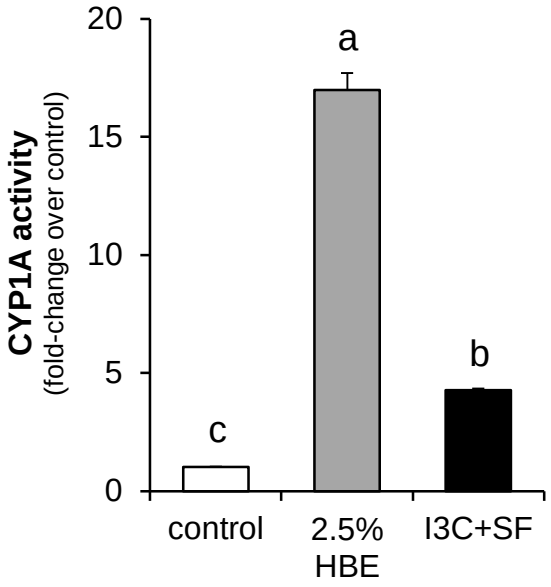


Figure 3.1. (continued)

C.



D.

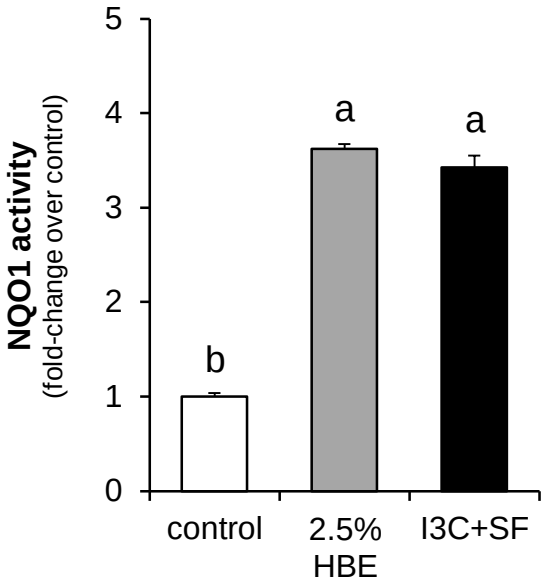


Figure 3.2. The effect of glucosinolate breakdown products on enzyme activity in chicken LMH cells. Cells were exposed to hydrolyzed broccoli extract (HBE, 2.5%), I3C (2.5-50 μM) or DIM (0.25-12.5 μM). Enzyme activity was measured after 48 h exposure time. Data, expressed as fold-change over control, represent mean ± SEM; n=3. Values that differ are indicated by different letters ($p < 0.05$).

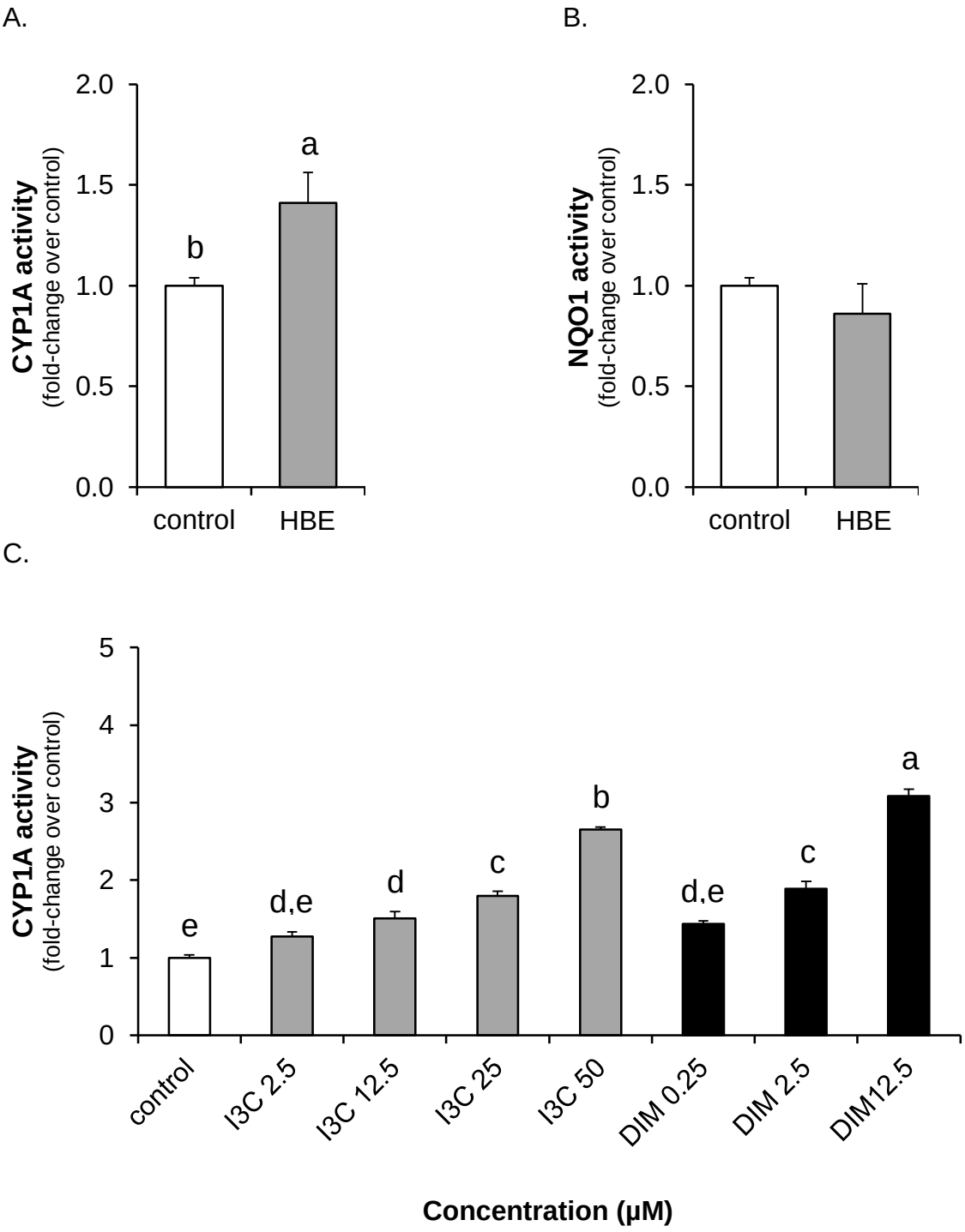


Figure 3.3. The effect of β-NF on (A) CYP1A activity and (B) NQO1 activity with or without inhibitors of CYP1A (CYP1A-i) or NQO1 (NQO1-i) in Hepa-1c1c7 cells. Cells were exposed to β-NF (1 μM) with or without CYP1A-i (10 μM) or NQO1-i (50 μM). Enzyme activity was measured after 24 h exposure time. Data, expressed as fold-change over control, represent mean ± SEM, n=4. Values that differ are indicated by different letters ($p < 0.05$).

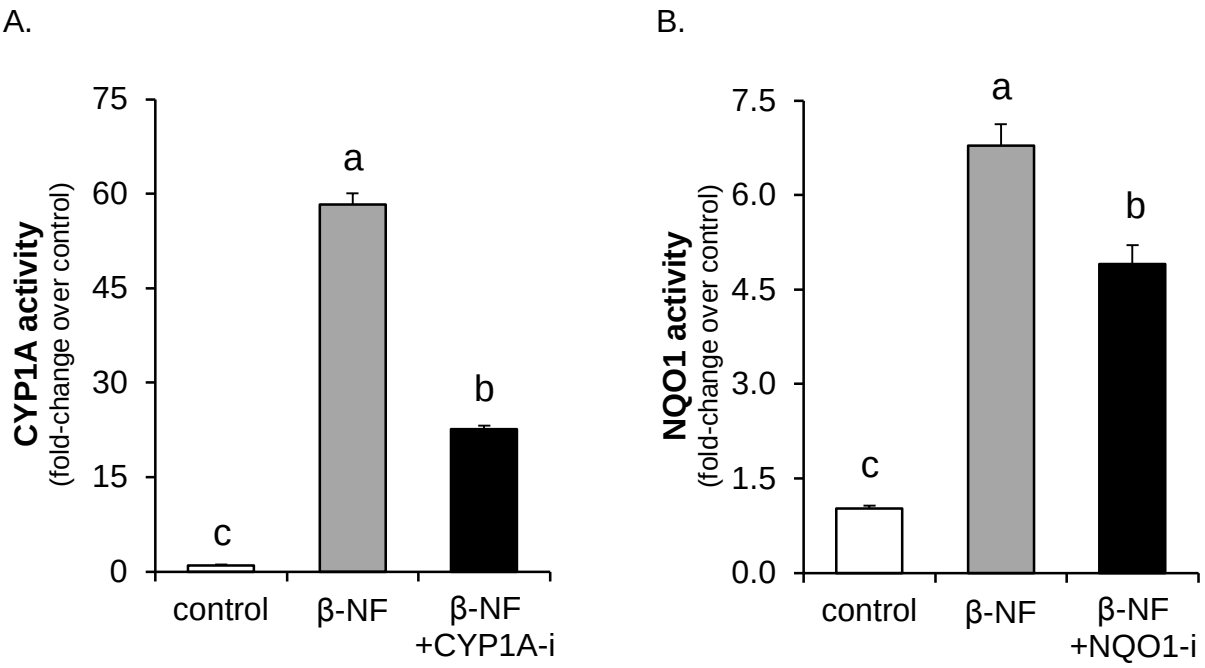


Figure 3.4. The effect of broccoli extract or β-NF on 2-methoxyestradiol formation in Hepa-1c1c7 cells. (A) Cells were pre-treated with hydrolyzed broccoli extract (HBE, 2.5%) for 24 h, followed by simultaneous exposure to 17β-estradiol (10 μM) and HBE (2.5%) for 24 h. (B) Cells were pre-treated with β-NF (1 μM) or β-NF+CYP1A-i (10 μM) for 24 h, followed by simultaneous exposure to 17β-estradiol (10 μM) and β-NF or β-NF+ CYP1A-i for 24 h. (C) Cells were pre-treated with β-NF (1 μM) or β-NF+NQO1-i (50 μM) for 24 h, followed by simultaneous exposure to 17β-estradiol (10 μM) and β-NF or β-NF +NQO1-i for 24 h. (D) Cells were pre-treated with β-NF (1 μM) or β-NF+COMT-i (3 μM) for 24 h, followed by simultaneous exposure to 17β-estradiol (10 μM) and β-NF or β-NF +COMT-i for 24 h. 2-Methoxyestradiol was extracted from all culture media and quantified by HPLC-MS/MS. Data, expressed as fold-change over control, represent mean ± SEM, n=3. Values that differ are indicated by different letters ($p < 0.05$). ND = below the level of detection.

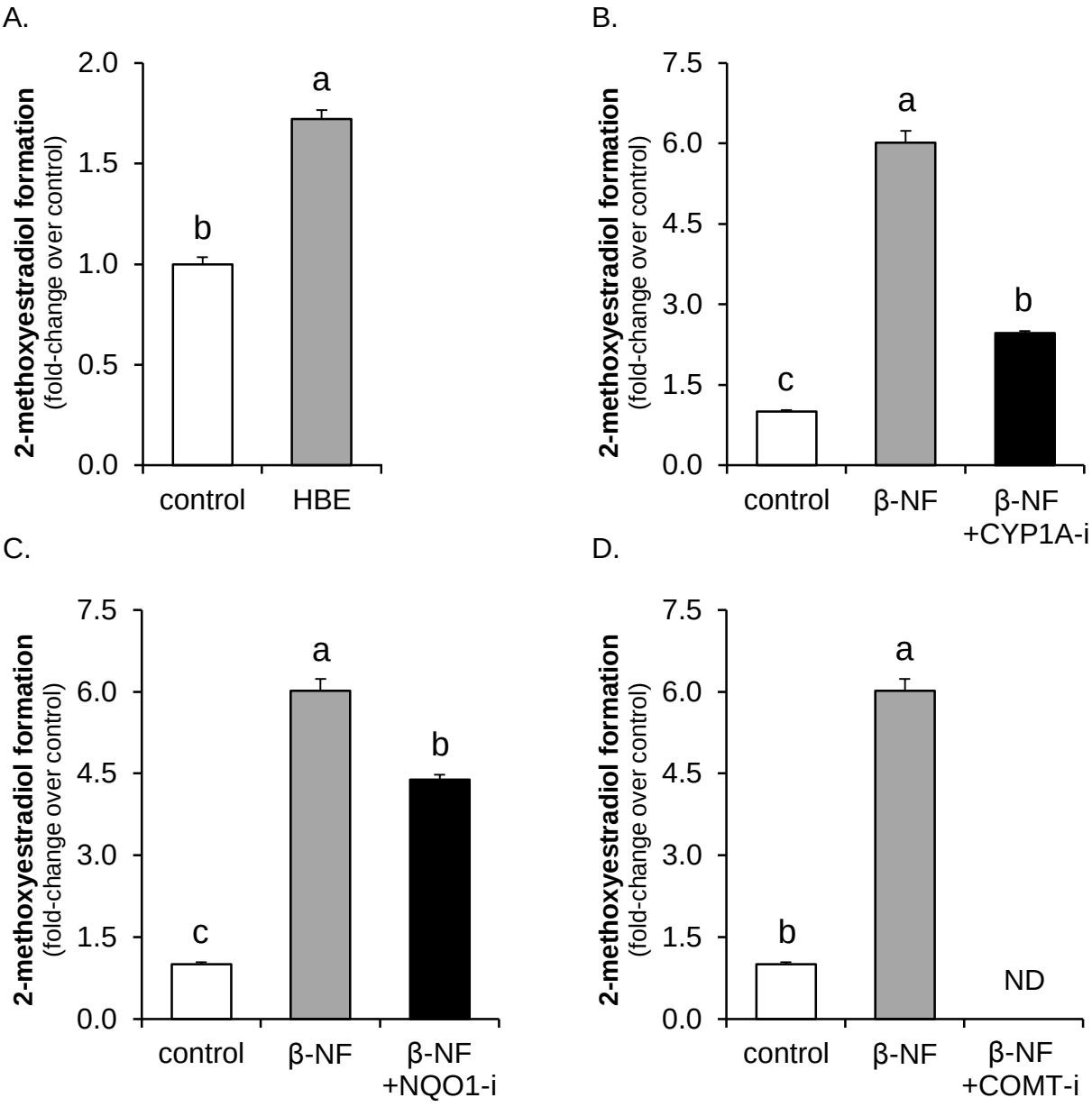


Figure 3.5. The effect of glucosinolate breakdown products on enzyme activity in (A,B) chicken liver and (C,D) ovary. Chickens were fed a diet supplemented with broccoli powder (10%) or I3C (2,500 ppm) for 7 days. CYP1A activity was measured in microsomal fractions; NQO1 activity was measured in cytosolic fractions. Data, expressed as fold-change over control, represent mean ± SEM, n=3. Values that differ are indicated by different letters ($p < 0.05$).

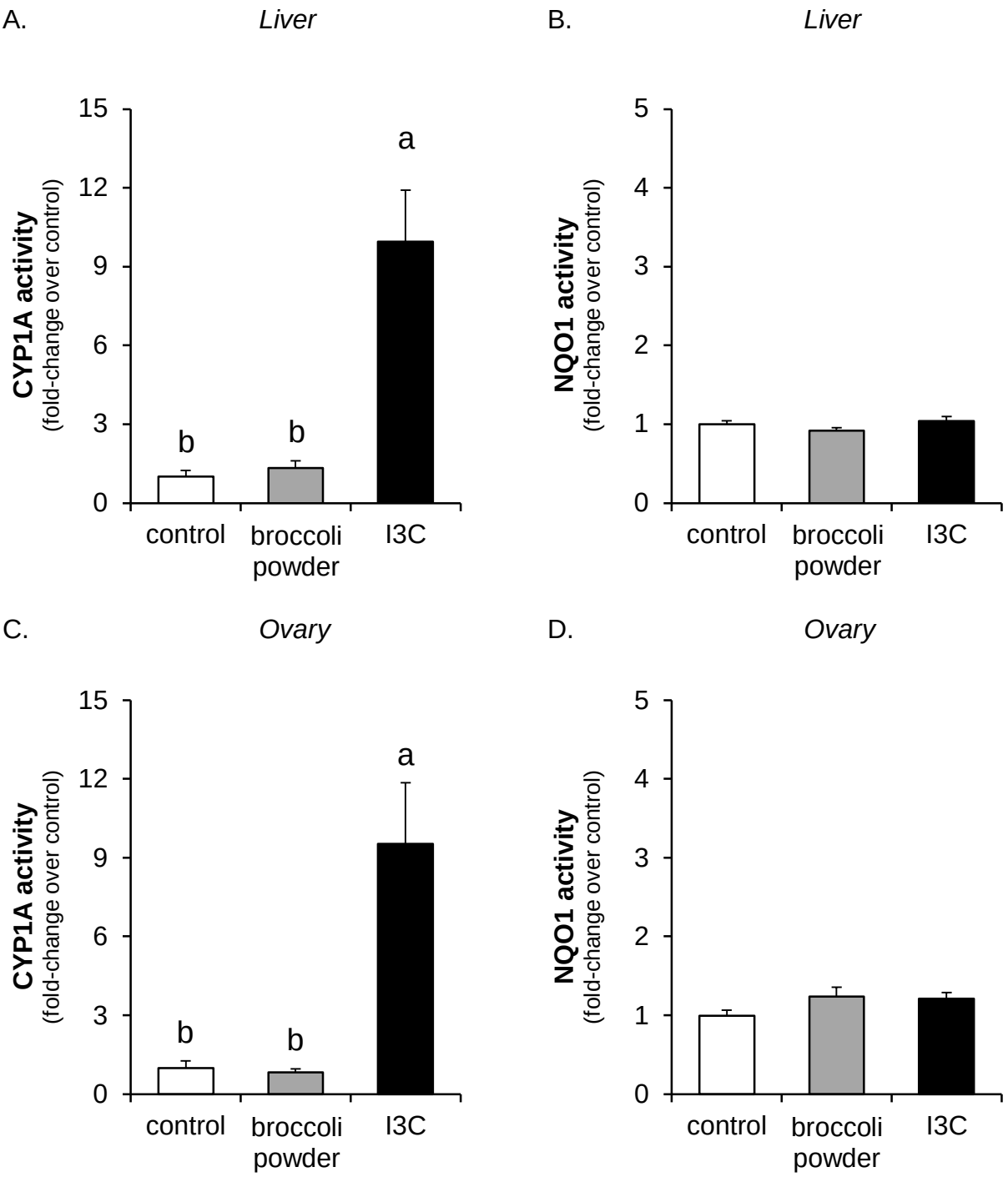
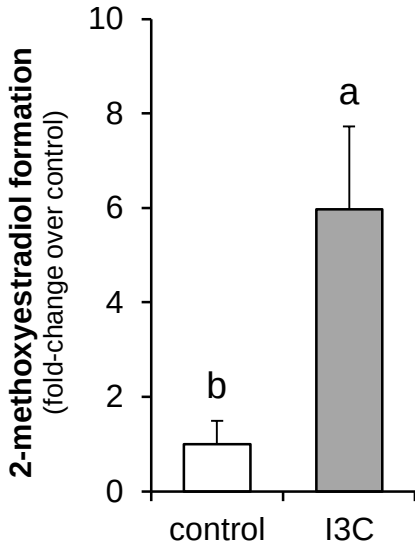


Figure 3.6. The effect of glucosinolate breakdown products on 2-methoxyestradiol formation in chicken liver. Chickens were fed a diet supplemented with I3C (2,500 ppm) for 7 days. Liver S9 fractions were incubated with 17β-estradiol (10 μM); 2-methoxyestradiol was extracted from the culture medium and quantified by HPLC-MS/MS. Data, expressed as fold-change over control, represent mean ± SEM, n=3. Values that differ are indicated by different letters ($p < 0.05$).



CHAPTER 4

Differential and interactive effects of broccoli bioactives on cancer cell growth and enzyme induction

4.1. Introduction

In Western societies, estrogen-associated cancers are a leading cause of mortality and morbidity in men and women (2). Cruciferous vegetables, such as broccoli, may offer greater protection against these cancers compared to other types of fruits and vegetables. This enhanced chemoprotective effect of crucifers, as observed for prostate (56, 206-208) and ovarian cancer (54, 212), is traditionally attributed to high levels of glucosinolates and their bioactive breakdown products (5, 8). A proposed mechanism of protection against estrogen-associated cancers by cruciferous vegetables is alteration of estrogen metabolism (9).

Glucosinolate hydrolysis products enhance formation of 2-methoxyestradiol in murine Hepa-1c1c7 hepatoma cells and chicken liver S9 fractions (Chapter 3). 2-Methoxyestradiol is an endogenous metabolite of 17β-estradiol with anticancer activity, formed as a result of the upregulation of the detoxification enzymes cytochrome P450 (CYP) 1A and NAD(P)H dehydrogenase, quinone 1 (NQO1) (Chapter 3). Estrogens are hydroxylated at the C-2 position by CYP1A to form 2-hydroxyestrogens (16), which are subsequently methylated to 2-methoxyestrogens by catechol-*O*-methyltransferase (COMT) (18), or oxidized further to a quinone (102). Reactive estrogen quinones can be reduced back to 2-hydroxyestrogens by NQO1 (103), again providing substrate for 2-methoxyestradiol formation (Fig. 2.5).

In Hepa-1c1c7 cells, a hydrolyzed extract of freeze-dried broccoli powder upregulates CYP1A activity to a greater extent than purified indole-3-carbinol (I3C) plus sulforaphane (SF) at concentrations equimolar to those present in the hydrolyzed broccoli extract (Chapter 3). Thus, we hypothesized that the growth-inhibitory effects of glucosinolate hydrolysis products are, in part, mediated via enhanced formation of 2-methoxyestradiol. We also hypothesized that the hydrolyzed broccoli extract shows greater inhibition of human prostate and ovarian cancer cell growth than purified I3C plus SF.

Induction of phase II detoxification enzymes, such as NQO1, is thought to play a major role in protection against cancer via enhanced inactivation and excretion of (potential) carcinogens (230, 231). The broccoli bioactives SF, quercetin and kaempferol have each been shown to upregulate NQO1 activity (136, 149, 232), but interactive effects of these compounds have not been investigated. Therefore, we hypothesized that combinations of SF plus quercetin, SF plus kaempferol or quercetin plus kaempferol show greater induction of NQO1 activity than any compound individually.

4.2. Methods

Plant material, chemicals and reagents. Broccoli florets (*Brassica oleracea* var. Monaco), freeze-dried and ground to a powder, were obtained from FutureCeuticals (Momence, IL) as described previously (21). Unless noted otherwise, chemicals and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Cell culture. Murine Hepa-1c1c7 hepatocellular carcinoma cells and human NIH:OVCAR-3 epithelial ovarian carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). The human LNCaP clone FGC epithelial prostate carcinoma cell line was a kind gift from Dr. J.W. Erdman, Jr., University of Illinois at Urbana-Champaign. Hepa-1c1c7 cells were maintained in Eagle's minimum essential medium with alpha modification, supplemented with 2.2 g/L sodium bicarbonate and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). LNCaP and OVCAR-3 cells were maintained in RPMI-1640 medium without phenol red, modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4,500 mg/L glucose, and 1,500 mg/L sodium bicarbonate, supplemented with 10% (LNCaP) or 20% fetal bovine serum and 0.01 mg/ml bovine insulin (OVCAR-3). Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Treatments. Indole-3-carbinol, SF, quercetin, kaempferol (LKT Laboratories, Inc. St. Paul, MN) and Ro 41-0960 (COMT inhibitor, COMT-i) were dissolved in DMSO; 17β-estradiol (Steraloids, Newport, RI) was dissolved in 95% ethanol. Compounds were sterile-filtered and stored at -20°C. Aqueous broccoli extract was prepared as described previously (Chapter 3) and added to the culture medium at 2.5-10% final concentration, equivalent to 1.25-5 mg broccoli powder per mL culture medium. All other treatments were added to the culture medium at 0.1% final concentration. The concentration of I3C in 2.5% hydrolyzed broccoli extract was 0.125 μM; the concentration of SF in the extract was 10-fold greater at 1.25 μM, as determined previously (21).

Enzyme activity. LNCaP and OVCAR-3 cells were seeded at 5 x 10^4 cells per well into flat bottom 96-well plates (Corning, Corning NY) and allowed to adhere for 48

h. After 48 h, medium was replaced with fresh medium containing 2.5% hydrolyzed broccoli extract (equivalent to 1.25 mg freeze-dried broccoli powder per mL culture medium). Hepa-1c1c7 cells were seeded at 1 x 10^4 cells per well into flat bottom 96-well plates (Corning) and allowed to adhere overnight. Medium was replaced with fresh medium containing purified SF (0.25-2 µM), quercetin (0.5-15 µM) or kaempferol (0.5-15 µM). Enzyme activity was determined after 24 h incubation.

The activity of CYP1A was measured as ethoxyresorufin *O*-deethylase activity (214); NQO1 activity was measured according to the method of Prochaska and Santamaria (215) with modification (216), both as described previously (Chapter 3).

Cell growth and caspase activity. LNCaP and OVCAR-3 cells were seeded at 2.5 x 10^3 cells per well into flat bottom 96-well plates (Corning) and allowed to adhere for 48 h. After 48 h, medium was replaced with estrogen-depleted medium (Cell Media Facility, University of Illinois at Urbana-Champaign), and cells were subsequently exposed to treatment for 96 h. 17β-Estradiol (10 nM) and the COMT inhibitor Ro 41-0960 (COMT-i, 3 µM) were added every 24h. Preliminary studies showed that in both cell lines, daily treatment with 10 nM 17β-estradiol for 96 h after 48 h of attachment significantly increased cell growth compared to untreated controls (data not shown). Daily treatment with 3 µM COMT-i, a dose that has previously been shown to abolish formation of 2-methoxyestradiol (Chapter 3), for 96 h after 48 h of attachment did not affect cell growth compared to untreated cells (data not shown). Hydrolyzed broccoli extract (2.5-10%), purified I3C (0.125 µM) and SF (1.25 µM) were added every 48 h. Preliminary studies showed that treatment with 2.5% hydrolyzed broccoli extract increased NQO1 activity, and that this level of activity was maintained for up to 48 h after addition of the treatment

(data not shown). The relative number of live cells, as a measure of cell growth, was determined by the CellTiter-Fluor™ Cell Viability Assay (Promega, Madison, WI), as per manufacturer's instruction. The activity of caspase 3 and 7, as a measure of apoptosis, was determined by the Caspase-Glo® 3/7 Assay (Promega), as per manufacturer's instruction. The number of live cells was determined after cells were allowed to adhere for 48 h, but prior to exposure to treatment. This measurement, labeled as "control", established the baseline number of cells for these experiments. The number of live cells was also determined after the 96 h treatment period.

Interaction analysis. Possible interactions between purified SF, quercetin and kaempferol on NQO1 activity were determined by comparing calculated (additive) and experimental (observed) values for various treatment conditions. Experimental values that were different from calculated values ($p < 0.05$) were defined as synergistic interactions (233).

Statistical analysis. Data were evaluated by one-way analysis of variance using SAS Statistical software (SAS Institute, Cary, NC), and values were determined to be different among treatments using Fisher's least significant difference procedure ($p < 0.05$). Individual pair-wise comparisons were performed using the two-sample *t*-test ($p < 0.05$).

4.3. Results

Effect of hydrolyzed broccoli extract on enzyme activity. The effect of hydrolyzed broccoli extract on CYP1A and NQO1 activity was determined in human prostate (LNCaP) and ovarian (OVCAR-3) carcinoma cells. Both cell lines were allowed to

adhere for 48 h, followed by a 24 h treatment period. In LNCaP cells, 2.5% hydrolyzed broccoli extract increased CYP1A and NQO1 activity 6.7- and 1.4-fold compared to untreated cells (Fig. 4.1A, B). In OVCAR-3 cells, 2.5% hydrolyzed broccoli extract modestly increased CYP1A and NQO1 activity 1.9- and 1.5-fold compared to untreated cells (Fig. 4.1C, D).

Effect of hydrolyzed broccoli extract on unstimulated or 17β-estradiol-stimulated cancer cell growth. The estrogen-responsive cell lines LNCaP and OVCAR-3 were grown in estrogen-depleted medium and allowed to adhere for 48 h (control), followed by a 96 h treatment period. Cells were exposed to increasing concentrations (2.5-10%) of hydrolyzed broccoli extract in the absence (Fig. 4.2A, 4.3A) or presence (Fig. 4.2B, 4.3B) of 17β-estradiol. In 17β-estradiol-stimulated cells, the effect of hydrolyzed broccoli extract was also determined in the presence of an inhibitor of COMT (Fig. 4.2C, 4.3C) The concentration of COMT-i (3μM) was chosen because it was previously shown to abolish formation of 2-methoxyestradiol (Chapter 3). 17β-estradiol and COMT-i were added every 24 h; hydrolyzed broccoli extract was were added every 48 h.

During the 96 h treatment period that followed 48 h to allow attachment, the number of unstimulated LNCaP cells (grown in the absence of 17β-estradiol) increased 5.3-fold compared to control. Treatment with hydrolyzed broccoli extract for 96 h decreased cell growth in a dose-dependent manner: compared to unstimulated cells, the extract inhibited cell growth by 49, 65 and 74% when 2.5, 5 or 10% extract was added, respectively. The number of live cells after treatment with 5 or 10% hydrolyzed broccoli extract for 96 h was no longer significantly greater than the number of live cells at the start of the 96 h treatment (control) (Fig. 4.2A).

Treatment of LNCaP cells with 17β-estradiol for 96 h after 48 h of attachment increased cell growth by 34% compared to cells that were grown for 96 h in the absence of 17β-estradiol. In the presence of 17β-estradiol, treatment with hydrolyzed broccoli extract for 96 h decreased cell growth dose-dependently: the extract inhibited cell growth by 10, 51 and 74% when 2.5, 5 or 10% extract was added, respectively, compared to cells that were exposed to 17β-estradiol only for 96 h (Fig. 4.2B)

In a similar experiment, LNCaP cells were co-treated with hydrolyzed broccoli extract and COMT-i in the presence of 17β-estradiol for 96 h after 48 h of attachment. Compared to cells that were exposed to 17β-estradiol only for 96 h, treatment with 17β-estradiol plus hydrolyzed broccoli extract for 96 h in the presence of COMT-i decreased cell growth in a dose-dependent manner: the extract inhibited cell growth by 31, 47 and 72% when 2.5, 5 or 10% extract was added, respectively (Fig. 4.2C).

Compared to control, the number of unstimulated OVCAR-3 cells (grown in the absence of 17β-estradiol) increased 4.5-fold during the 96 h treatment period that followed 48 h to allow attachment. Treatment with hydrolyzed broccoli extract for 96 h decreased cell growth in a dose-dependent manner: compared to unstimulated cells, the extract inhibited cell growth by 57, 84 and 85% when 2.5, 5 or 10% extract was added, respectively. The number of live cells after treatment with 5 or 10% hydrolyzed broccoli extract for 96 h was no longer significantly greater than the number of live cells at the start of treatment (control) (Fig. 4.3A).

Treatment of OVCAR-3 cells with 17β-estradiol for 96 h after 48 h of attachment increased cell growth by 31% compared to cells that were grown for 96 h in the absence of 17β-estradiol. In the presence of 17β-estradiol, treatment with hydrolyzed broccoli

extract for 96 h decreased cell growth dose-dependently: the extract inhibited cell growth by 65, 82 and 92% when 2.5, 5 or 10% extract was added, respectively, compared to cells that were exposed to 17β-estradiol only for 96 h. The number of live cells after treatment with 5 or 10% hydrolyzed broccoli extract for 96 h was no longer significantly greater than the number of live cells at the start of treatment (control) (Fig. 4.3B).

In a similar experiment, OVCAR-3 cells were co-treated with hydrolyzed broccoli extract and COMT-i in the presence of 17β-estradiol for 96 h after 48 h of attachment. Compared to cells that were exposed to 17β-estradiol only for 96 h, treatment with hydrolyzed broccoli extract for 96 h in the presence of COMT-i decreased cell growth in a dose-dependent manner: the extract inhibited cell growth by 78, 80 and 87% when 2.5, 5 or 10% extract was added, respectively. The number of live cells after each treatment with hydrolyzed broccoli extract for 96 h was no longer significantly greater than the number of live cells at the start of treatment (control) (Fig. 4.3C).

Effect of hydrolyzed broccoli extract, I3C and SF on cancer cell growth. LNCaP and OVCAR-3 cells were grown in estrogen-depleted medium and allowed to adhere for 48 h (control), followed by a 96 h treatment period. Cells were exposed to purified I3C (0.125 μM), SF (1.25 μM), I3C plus SF, or 2.5% hydrolyzed broccoli extract for 96 h.

In LNCaP cells, following 48 h of attachment, 96 h treatment with purified I3C, SF or I3C plus SF did not alter cell growth compared to cells grown in the absence of treatment. In contrast, exposure to hydrolyzed broccoli extract for 96 h decreased cell growth by 51% compared to cells grown in the absence of treatment (Fig. 4.4A).

In OVCAR-3 cells, after 48 h of attachment, 96 h treatment to purified I3C or SF did not alter cell growth compared to cells grown in the absence of treatment. Exposure to purified I3C plus SF decreased cell growth by 27%, and exposure to hydrolyzed broccoli extract decreased cell growth by 58% compared to cells grown in the absence of treatment (Fig. 4.4B).

Effect of hydrolyzed broccoli extract, I3C and SF on caspase activity. LNCaP and OVCAR-3 cells were grown in estrogen-depleted medium and allowed to adhere for 48, followed by a 96 h treatment period. Cells were exposed to 2.5% hydrolyzed broccoli extract or purified I3C (0.125 μM) plus SF (1.25 μM) for 96 h.

In LNCaP cells, after 48 h of attachment, 96 h treatment with hydrolyzed broccoli extract increased activity of caspase 3 and 7 1.9-fold compared to cells grown in the absence of treatment. No increase in caspase activity was observed after 96 h treatment with I3C plus SF compared to cells grown in the absence of treatment (Fig. 4.5A).

In OVCAR-3 cells, after 48 h of attachment, 96 h treatment with hydrolyzed broccoli extract increased activity of caspase 3 and 7 2.5-fold compared to cells grown in the absence of treatment. No increase in caspase activity was observed after 96 h treatment with I3C plus SF compared to cells grown in the absence of treatment (Fig. 4.5B).

Dose-dependent effects of SF, quercetin and kaempferol on NQO1 activity. Hepa-1c1c7 cells were allowed to adhere for 24 h, followed by a 24 h treatment period. Cells were exposed to increasing concentrations of SF (0.25-2 μM), quercetin (0.5-15 μM) or kaempferol (0.5-15 μM). Sulforaphane, quercetin and kaempferol individually

induced NQO1 activity in a dose-dependent manner (Fig. 4.6A-C). At 1.25 μM, SF increased NQO1 activity by 190% compared to untreated cells (control); exposure to 2 μM SF did not further increase enzyme activity (Fig. 4.6A). At 5 μM, quercetin increased NQO1 activity by 93% compared to control; exposure to higher concentrations of quercetin decreased enzyme activity (Fig. 4.6B). At 5 μM, kaempferol increased NQO1 activity by 62% over control; exposure to higher concentrations of kaempferol did not further increase enzyme activity (Fig. 4.6C).

Interactive effects among SF, quercetin and kaempferol on NQO1 activity. To determine interactive effects among SF, quercetin and kaempferol on NQO1 activity, individual doses were selected that provided moderate enzyme induction (~20% of maximum activity). The rationale for selecting these doses was to preventreaching maximum enzyme induction with combined treatments. The selected doses were: SF 0.1 μM, quercetin 1 μM, and kaempferol 1 μM.

Alone, SF at 0.1 or 0.75 μM increased enzyme activity by 69 and 155% over control; quercetin at 1 or 1.75 μM increased activity by 9 and 17% (Fig. 4.7A). When combined, SF plus quercetin upregulated NQO1 activity to 83% (SF 0.1 μM + Q 1 μM), 162% (SF 0.1 μM + Q 1.75 μM) and 201% (SF 0.75 μM + Q 1.75 μM) over control. At low doses, the observed effect of SF plus quercetin combined (SF 0.1 μM + Q 1 μM) did not differ from the calculated additive effect. At higher doses, the effect of SF plus quercetin combined (SF 0.1 μM + Q 1.75 μM, and SF 0.75 μM + Q 1.75 μM) was greater than each respective calculated additive effect (Fig. 4.7A).

Kaempferol at 0.5 or 1 μM had no effect on NQO1 activity compared to control (Fig. 4.7B). When combined, SF plus kaempferol increased NQO1 activity to 94% (SF

0.1 μM + K 1 μM), 210% (SF 0.75 μM + K 0.5 μM) and 273% (SF 0.75 μM + K 1 μM) over control. Each combination of SF plus kaempferol had a greater effect on NQO1 activity than SF alone at the equivalent dose (Fig. 4.7B).

Quercetin and kaempferol combined increased NQO1 activity to 18% (Q 1 μM + K 0.5 μM), 37% (Q 1.75 μM + K 0.5 μM) and 49% (Q 1.75 μM + K 1 μM) over control. Each combination of quercetin plus kaempferol had a greater effect on NQO1 activity than quercetin alone at the equivalent dose (Fig. 4.7C).

4.4. Discussion

This study demonstrates for the first time that an aqueous extract of freeze-dried broccoli powder inhibits growth of human prostate (LNCaP) and ovarian (OVCAR-3) cancer cells, and concomitantly increases activity of caspase 3 and 7 activity. The extract, at the effective dose, contained a physiologically relevant level of SF (1.25 μM) that can be obtained in the plasma after consumption of a broccoli-rich meal (234-236). The study also shows for the first time interactive effects among SF, quercetin and kaempferol NQO1 activity in the murine Hepa-1c1c7 hepatoma cell line.

The hydrolyzed broccoli extract at 2.5% enhances formation of 2-methoxyestradiol, an endogenous metabolite of 17β-estradiol, via upregulation of the detoxification enzymes CYP1A and NQO1 (Chapter 3). 2-Methoxyestradiol has been shown to inhibit cell growth and induce apoptosis in human prostate (108, 109) and ovarian (106, 117) cancer cell lines. In this study, the hydrolyzed broccoli extract increased CYP1A and NQO1 activity (Fig. 4.2), and inhibited cell growth in the presence of 17β-estradiol in both human cancer cell lines (Fig. 4.2B and 4.3C). To determine

whether this decrease in cell growth was due to increased formation of 2-methoxyestradiol, cell were co-treated with the COMT inhibitor Ro 41-096 at a dose that has previously been shown to abolish formation of 2-methoxyestradiol (Chapter 3). Co-treatment with the COMT inhibitor did not attenuate the inhibitory effect of the hydrolyzed broccoli extract in either cell line (Fig. 4.2C and 4.3C). Furthermore, hydrolyzed broccoli extract inhibited growth of both cell lines to a similar extent in the absence (Fig. 4.2A and 4.3A) or presence of 17β-estradiol (Fig. 4.2B and 4.3B). These results suggest that, in this study, the observed inhibition of cancer cell growth is not a result of increased formation of 2-methoxyestradiol. A possible explanation may be that only a small amount of 2-methoxyestradiol was formed from 17β-estradiol in LNCaP and OVCAR-3 cells exposed to hydrolyzed broccoli extract, and that this amount was too small to overcome the growth stimulatory effects of 17β-estradiol. Follow-up studies involving stronger inducers of CYP1A and NQO1, such as β-naphthoflavone, or overexpression of these enzymes in target cells, are necessary to determine whether increased formation of endogenous 2-methoxyestradiol has therapeutic potential in the treatment of estrogen-associated cancers.

Co-treatment with the COMT inhibitor did not alleviate inhibition of cancer cell growth by the hydrolyzed broccoli extract (Fig. 4.2C and 4.3C). Thus, it can be concluded that the growth-inhibitory effects of glucosinolate hydrolysis products are mediated via biological pathways other than estrogen metabolism. Based on *in vitro* studies involving purified bioactive compounds, these include induction of cell cycle arrest and apoptosis via suppression of cell survival pathways and an increase of proapoptotic regulators, as discussed in Chapter 2. However, most studies evaluate

only one bioactive compound at a time, and often at concentrations that far exceed what can be obtained in the plasma through dietary means. In addition, the majority of purified bioactives are available in their aglycone form, whereas in the plant most bioactives are present as conjugated precursors. More studies that involve bioactives as part of the whole plant are necessary to identify which compounds and which molecular targets are relevant for the prevention of cancer development in humans.

The enhanced chemoprotective effect of cruciferous vegetables, as observed for prostate (56, 206-208) and ovarian cancer (54, 212) is traditionally attributed to high levels of glucosinolates and their breakdown products (5, 8). In this study, the inhibitory effect of a hydrolyzed broccoli extract on human prostate and ovarian cancer cell growth was compared to the effect of the purified glucosinolate breakdown products I3C and SF. The hydrolyzed broccoli extract inhibited growth of both cell lines to a greater extent than combinations of purified I3C plus SF at concentrations equimolar to those found in the extract (Fig. 4.4). When evaluated individually, neither purified I3C nor SF affected growth in either cell line (Fig 4.4). These results indicate that inhibition of cell growth by the broccoli extract is not fully accounted for by I3C and SF.

Indole-3-carbinol and SF have each been shown to induce apoptosis in human prostate and ovarian cancer cell lines (88, 91, 203, 237, 238), although most studies used supra-physiological doses. This study determined the effect of a hydrolyzed broccoli extract on caspase activity in human prostate and ovarian cancer cells, compared to a combination of purified I3C plus SF at concentrations equimolar to the extract. In both cell lines, the hydrolyzed broccoli extract increased activity of caspases 3 and 7, whereas the combination of purified I3C plus SF did not have an effect on

caspase activity in either cell line (Fig. 4.5). These data suggest that the hydrolyzed broccoli extract inhibits growth of LNCaP and OVCAR-3 cells via induction of apoptosis, but that I3C and SF present in the extract do not fully account for this effect. It is concluded that additional compounds in the hydrolyzed broccoli extract, alone or in combination with I3C and/or SF, are responsible for the enhanced growth inhibition and induction of caspase activity.

Caspases 3 and 7 play a central role in the late, execution phase of apoptosis and can be activated via the intrinsic pathway (irradiation, genetic and/or metabolic stress) as well as the extrinsic, receptor-mediated pathway (239). Whether glucosinolate hydrolysis products, as part of the whole food, induce apoptosis via the intrinsic and/or extrinsic pathway, remains to be determined.

Inactivation and subsequent excretion of potentially carcinogenic compounds by phase II detoxification enzymes is thought to play a major role in protection against cancer (230, 231). Sulforaphane, quercetin and kaempferol have each been shown to increase activity of NQO1 (136, 149, 232), a key enzyme involved in the cellular defense against oxidative stress (240). This study examined the interactive effects between SF, quercetin and kaempferol on NQO1 activity using the murine Hepa-1c1c7 hepatoma cell line.

Sulforaphane and quercetin, but not kaempferol, individually increased NQO1 activity at the levels tested. The impact of SF on enzyme activity was much greater at low doses compared to quercetin, indicating that SF is a more potent inducer of NQO1 activity than quercetin (Fig. 4.7A).

Each combination of SF plus quercetin increased NQO1 activity to a greater extent than the individual compounds. At low dose combinations, the observed effect of SF plus quercetin combined did not differ from the calculated additive effect. At higher dose combinations of SF plus quercetin, the observed increase in enzyme activity was greater than the calculated effect, which indicates a synergistic interaction (Fig. 4.7A).

Although kaempferol individually did not have an impact on NQO1 activity, combined treatments with SF or quercetin increased enzyme activity compared to SF and quercetin alone (Fig. 4.7B, C). For each dose combination of SF plus kaempferol, or quercetin plus kaempferol, the observed increase in enzyme activity was greater than the calculated additive effect, which is indicative of synergistic interactions (Fig. 4.7A-C).

Taken together, these results indicate that SF individually is a much more potent inducer of NQO1 activity than quercetin or kaempferol. Combined, these compounds interact to increase NQO1 activity in a synergistic manner. Possible mechanisms underlying the observed synergistic effects among SF, quercetin and kaempferol on NQO1 activity are discussed below.

Upregulation of NQO1 activity by SF is dependent on the transcription factor Nrf2. (71, 241, 242). Under homeostatic conditions, Nrf2 is anchored to the cytoplasmic protein Keap1 and is constitutively degraded via the ubiquitin-proteasome pathway. Under conditions of oxidative stress, or in the presence of chemoprotective compounds such as SF, the Nrf2-Keap1 complex becomes unstable. This results in increased translocation of Nrf-2 into the nucleus and subsequent activation of antioxidant response element (ARE)-containing target genes, including NQO1 (65, 66). Sulforaphane has been shown to increase Nrf2 protein levels and to enhance

translocation of Nrf2 into the nucleus of human keratinocytes (243). In the human HepG2 hepatoma cell line, SF has been shown to stabilize Nrf2, presumably via Keap1-SF adduct formation (69). Similar effects of quercetin on Nrf2 levels and increased Keap-1 turnover have been observed for quercetin (244). Kaempferol has been shown to increase translocation of Nrf2 into the nucleus of mouse auditory cells (245). These overlapping mechanisms of enhanced Nrf-2 stabilization and translocation may lead to the synergistic upregulation of NQO1 activity by combinations of SF, quercetin and kaempferol.

The bifunctional inducer I3C has been shown to induce NQO1 transcription and activation in a synergistic manner when combined with crambene, a nitrile product of the glucosinolate progoitrin. The observed synergy at the transcriptional level is thought to be the result of combined activation of the xenobiotic response element (XRE) and the ARE in the regulatory region of the gene by I3C and crambene, respectively (246). Quercetin and kaempferol have both been shown to increase transcription of CYP1A1, presumably via activation of the XRE (247). Thus, combined activation of the XRE by quercetin and kaempferol, and the ARE by SF could also play a role in the synergistic upregulation of NQO1 activity.

Dietary bioactive components have been shown to alter the efficacy of pharmacological agents not only through modulation of detoxification enzymes (248, 249), but also through interaction with efflux transporter systems, which may alter the intracellular dose and rate of excretion of a compound (250, 251). Exposure to SF leads to its intracellular accumulation, followed by conjugation to glutathione and rapid excretion from the cell (252). The efflux of SF and its glutathione conjugate appears to

be mediated by the membrane transporters P-glycoprotein (P-gp) and multidrug resistance protein 1 (MRP1) (252, 253). Quercetin has been shown to decrease expression and activity of P-gp, MRP1 and MRP2 *in vitro* (254-256, 257). Kaempferol has been demonstrated to decrease MRP-mediated efflux in multidrug-resistant cancer cells (258) and inhibit cellular export of quercetin (259). Taken together, these data suggest that combinations of SF quercetin and kaempferol can enhance the intracellular concentration of the individual components via inhibition of transporter-mediated efflux, resulting in synergistic upregulation of NQO1 activity.

The levels of quercetin and kaempferol in the hydrolyzed broccoli extract have not been determined. Based on an analysis of 80 commercial broccoli samples (260), the amount of quercetin in 2.5% hydrolyzed broccoli extract is expected to be between 0.01-5 μM, and the amount of kaempferol between 0.1-6 μM. At these levels, quercetin could have an impact on cancer cell growth, but the levels of kaempferol may be too low to have an effect. Additional bioactive compounds may be present in the hydrolyzed broccoli extract at concentrations that, alone or in combination, inhibit cell growth. Of particular interest is selenium, whose accumulation in cruciferous vegetables has been shown to enhance their chemoprotective potential (261).

The doses of quercetin and kaempferol that, alone or in combination, had an effect on NQO1 activity can be expected to be present in the hydrolyzed broccoli extract. However, it was previously shown that the broccoli extract did not enhance NQO1 activity compared to purified SF at equimolar concentrations (Chapter 3), suggesting a maximum increase in NQO1 activity had been reached. Furthermore, although much of the literature evaluates activity of quercetin and kaempferol in their

respective aglycone forms, the broccoli extract, even after thio-hydrolysis, may still contain *O*-glycosides of quercetin and kaempferol. The concentration and chemical form of quercetin and kamepferol in hydrolyzed broccoli remains to be determined.

In conclusion, this study showed that broccoli bioactives, at concentrations that can be obtained in the plasma through dietary means, inhibit growth of prostate and ovarian cancer cells *in vitro*, possibly via induction of apoptosis. The chemoprotective effect of broccoli cannot solely be attributed to glucosinolate hydrolysis products: other bioactive components of broccoli, such as polyphenolic compounds, may contribute to this effect. Sulforaphane, quercetin and kaempferol were shown to increase NQO1 activity in an additive or even synergistic manner at concentrations that can be reached in the plasma, thus making these interactions physiologically relevant. Therefore, possible interactions between multiple bioactive components should be taken into consideration when investigating the mechanisms underlying the health-promoting properties of broccoli, or other whole foods.

4.5. Figures

Figure 4.1. The effect of hydrolyzed broccoli extract (HBE) on enzyme activity in (A, B) human LNCaP prostate carcinoma cells and (C, D) human OVCAR-3 ovarian carcinoma cells. Cells were exposed to 2.5% (HBE; enzyme activity was measured after 24 h exposure time. Data, expressed as fold-change over control, represent mean ± SEM, n=4. Values that differ from control are indicated by an asterisk (*, $p < 0.05$).

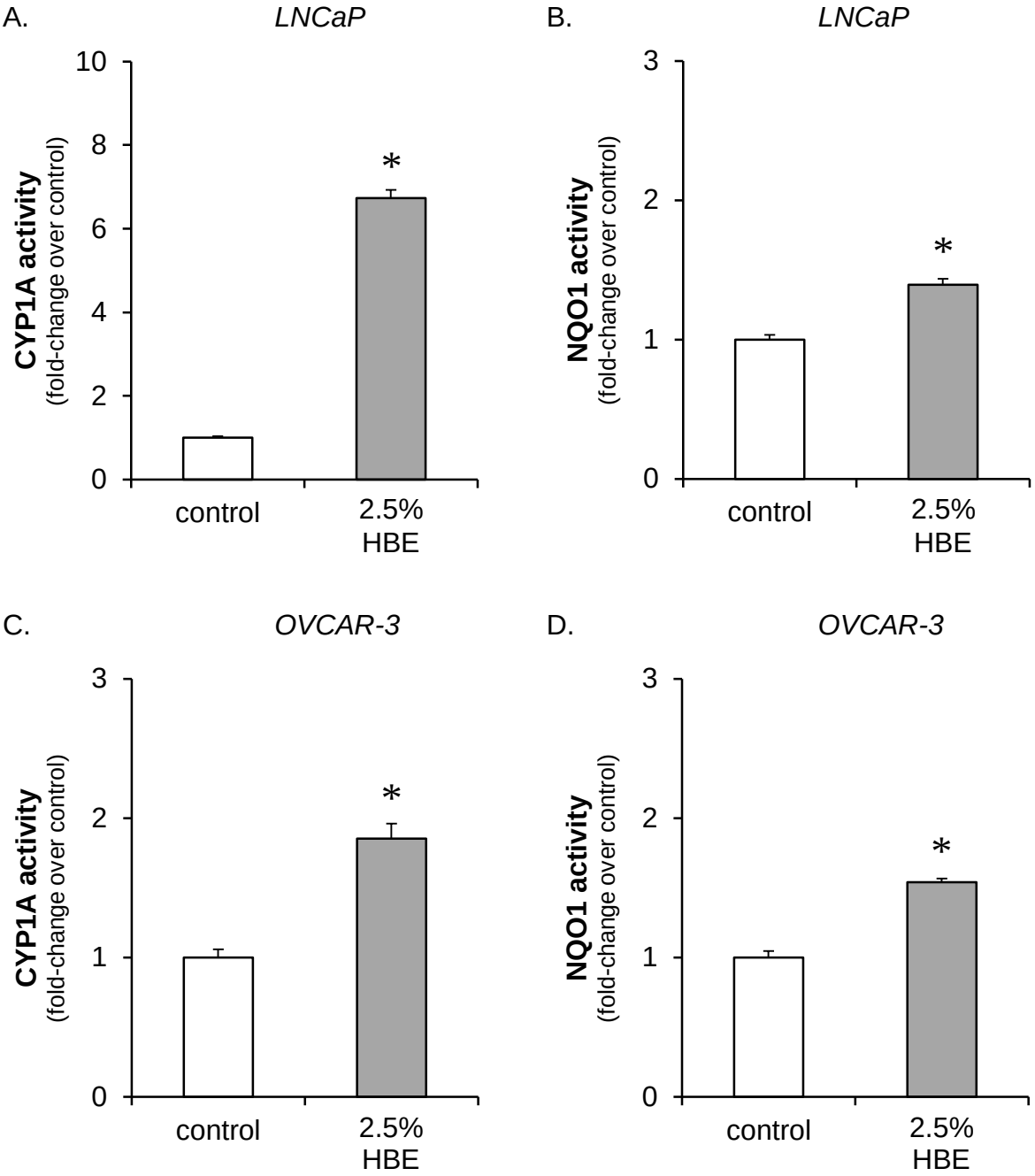
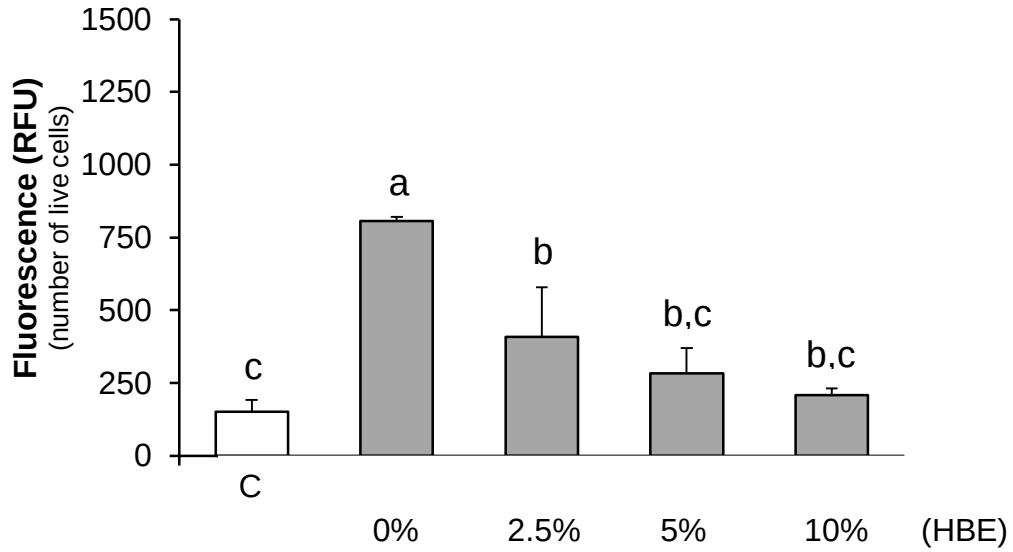


Figure 4.2. The effect of hydrolyzed broccoli extract (HBE) on cell growth in unstimulated or 17β-estradiol-stimulated LNCaP cells. After 48 h attachment, LNCaP cells were treated for 96 h with 2.5-10% HBE in (A) the absence or (B) presence of 10 nM 17β-estradiol (E_2), or (C) the presence of both17β-estradiol and 3 μM COMT inhibitor (COMT-i). 17β-Estradiol and COMT-i were added every 24 h; HBE was added every 48 h. The number of live cells was determined after 48 h attachment, but prior to treatment (control, C), and after the 96 h treatment period. Data, expressed as fluorescence, represent mean ± SEM, n=3. Values that differ are indicated by different letters ($p < 0.05$).

A.



B.

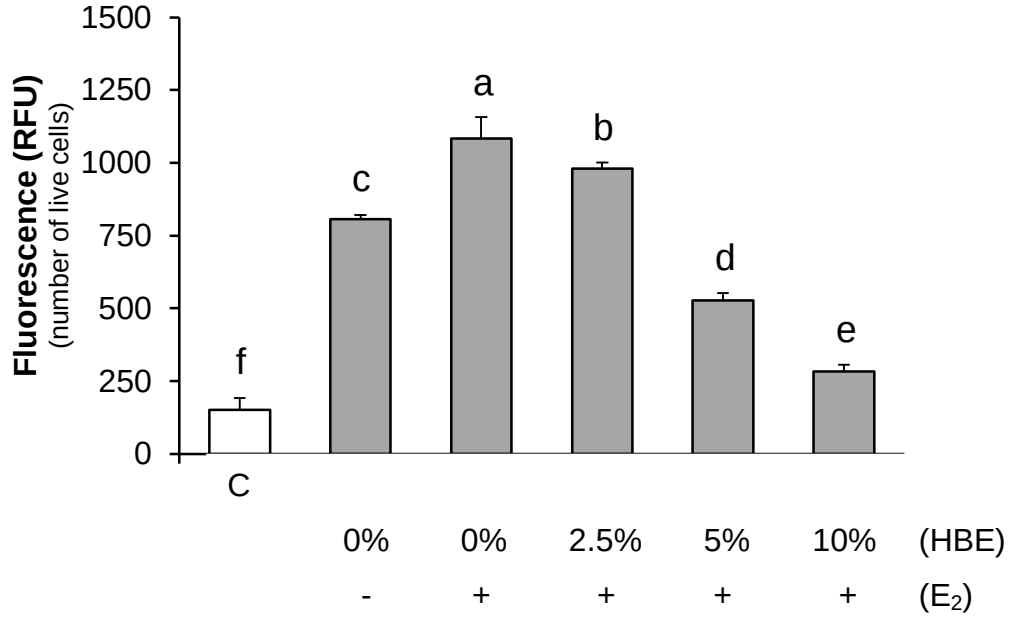


Figure 4.2. (continued)

C.

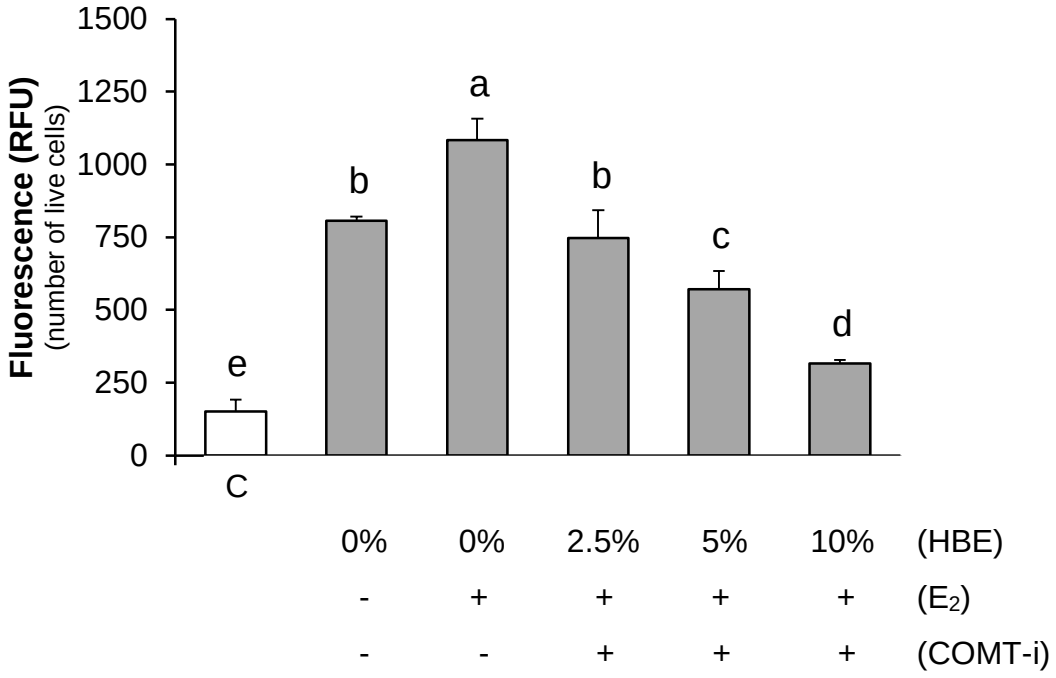
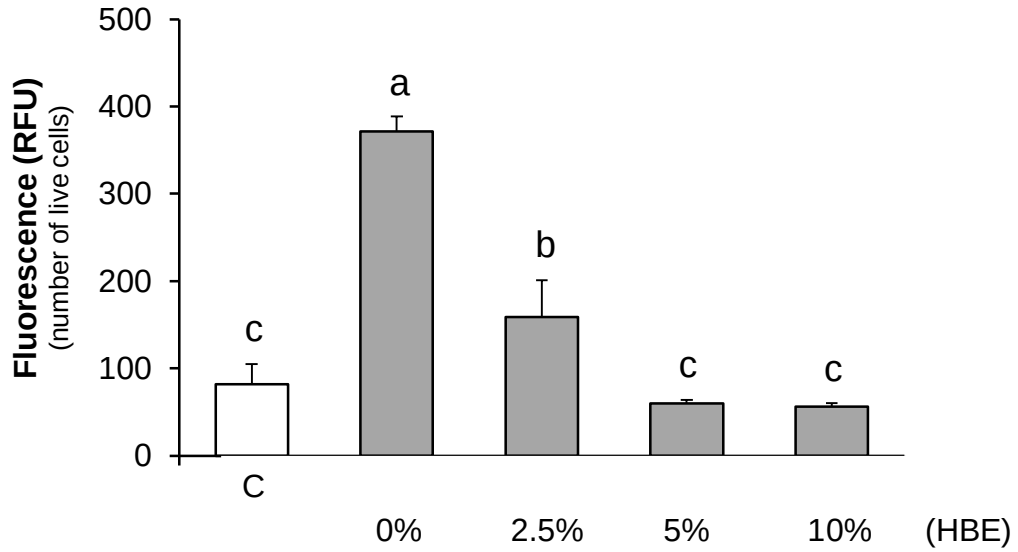


Figure 4.3. The effect of hydrolyzed broccoli extract (HBE) on cell growth in unstimulated or 17β-estradiol-stimulated OVCAR-3 cells. After 48 h attachment, OVCAR-3 cells were treated for 96 h with 2.5-10% HBE in (A) the absence or (B) presence of 10 nM 17β-estradiol (E_2), or (C) the presence of both17β-estradiol and 3 μM COMT inhibitor (COMT-i). 17β-Estradiol and COMT-i were added every 24 h; HBE was added every 48 h. The number of live cells was determined after 48 h attachment, but prior to treatment (control, C), and after the 96 h treatment period. Data, expressed as fluorescence, represent mean ± SEM, n=3. Values that differ are indicated by different letters ($p < 0.05$).

A.



B.

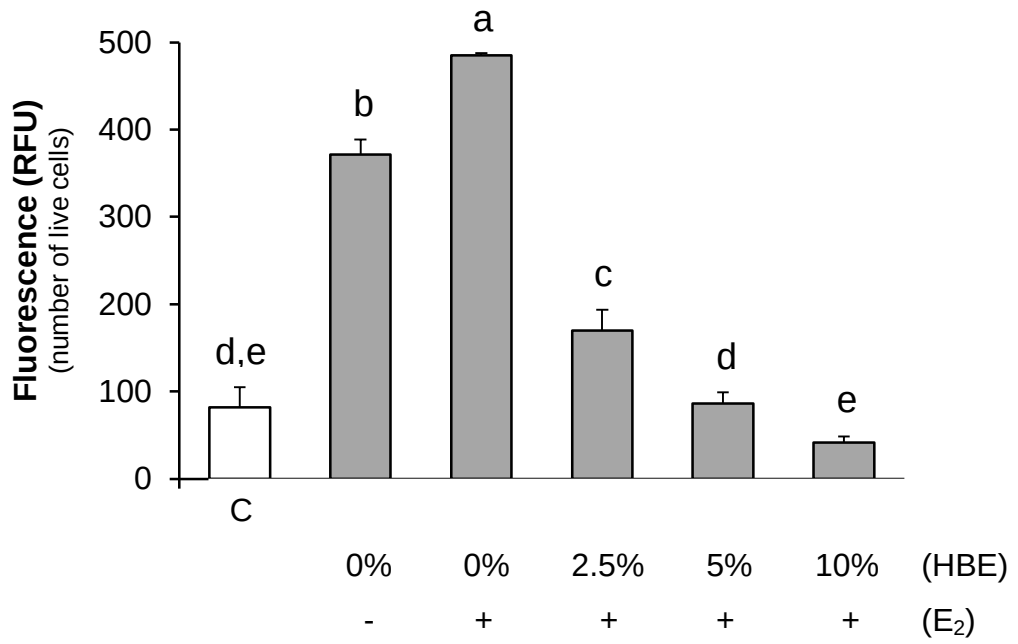


Figure 4.3. (continued)

C.

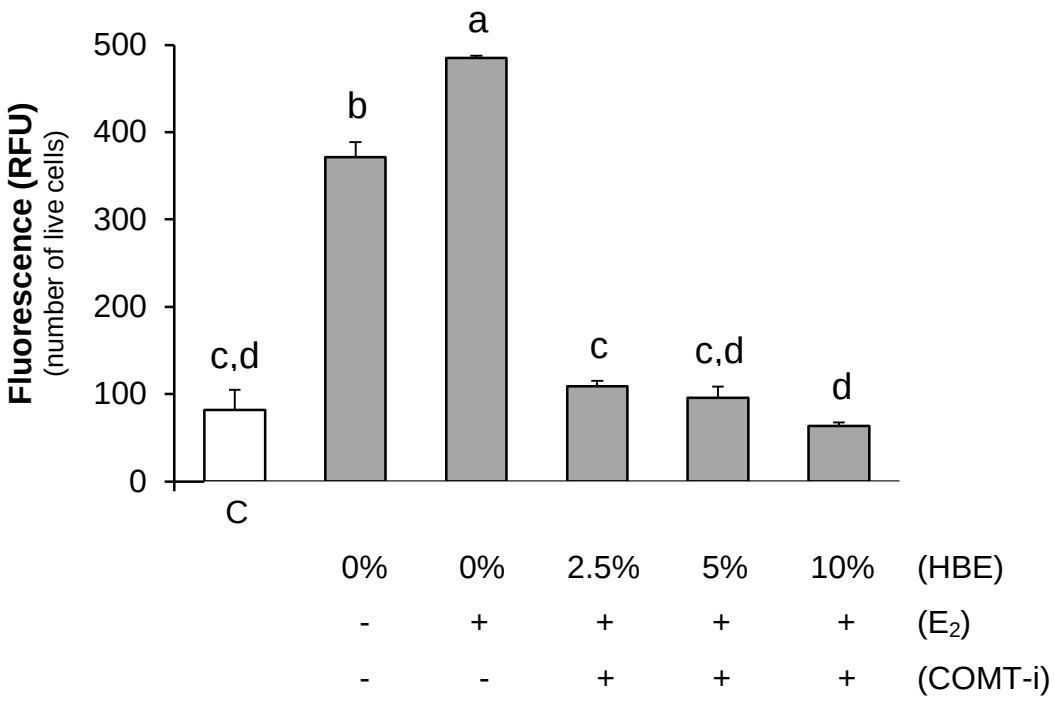


Figure 4.4. The effect of hydrolyzed broccoli extract (HBE), I3C and SF on growth of (A) LNCaP and (B) OVCAR-3 cells. After 48 h attachment, cells were treated for 96 h with purified I3C (0.125 µM), SF (1.25 µM), I3C plus SF or 2.5% HBE. Treatments were added every 48 h. The number of live cells was determined after 48 h attachment, but prior to treatment (control, C), and after the 96 h treatment period. Data, expressed as fluorescence, represent mean ± SEM, n=3. Values that differ are indicated by different letters ($p < 0.05$). No Tx: no treatment added after 48 h attachment.

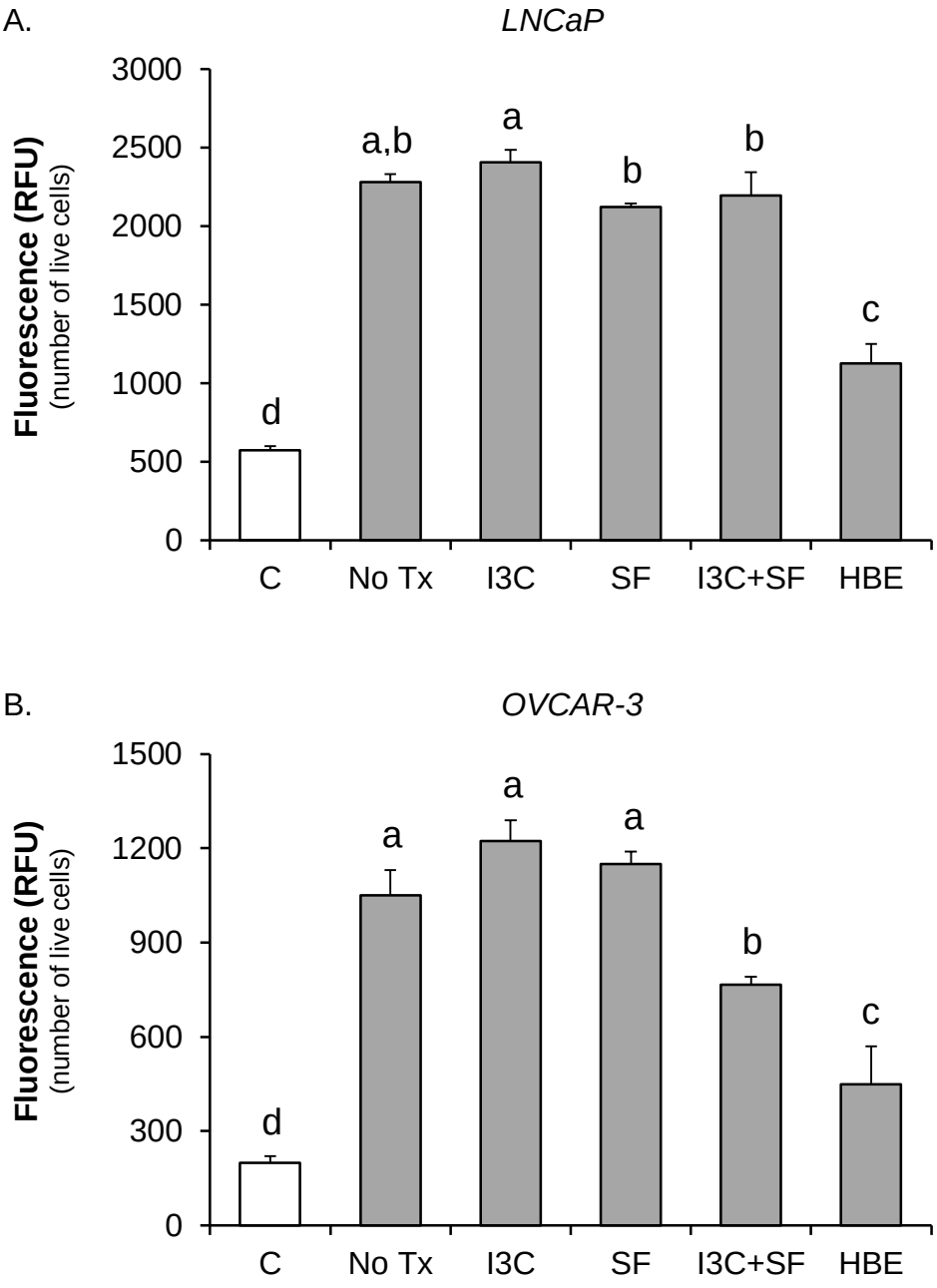
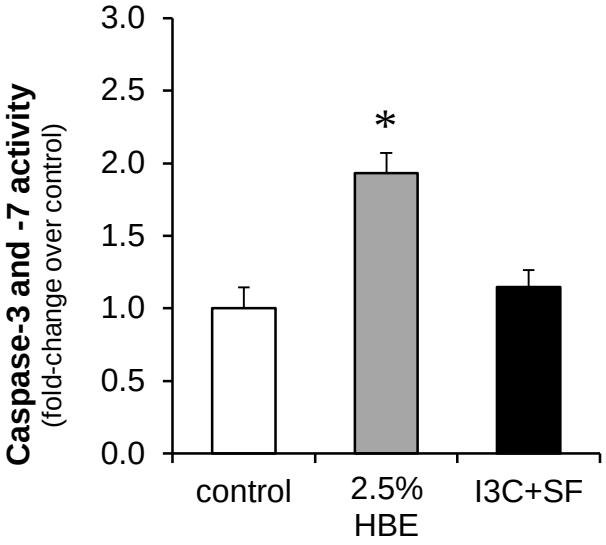


Figure 4.5. The effect of hydrolyzed broccoli extract (HBE) or I3C plus SF on caspase activity in (A) LNCaP or (B) OVCAR-3 cells. After 48 h attachment, cells were treated for 96 h with 2.5% HBE or purified I3C (0.125 μM) plus SF (1.25 μM).Treatments were added every 48 h. Activity of caspase 3 and 7 was determined after the 96 h treatment period. Data, expressed as fold-change over control, represent mean ± SEM, n=3; values that differ from control are indicated by an asterisk (*, $p < 0.05$).

A. *LNCaP*



B. *OVCAR-3*

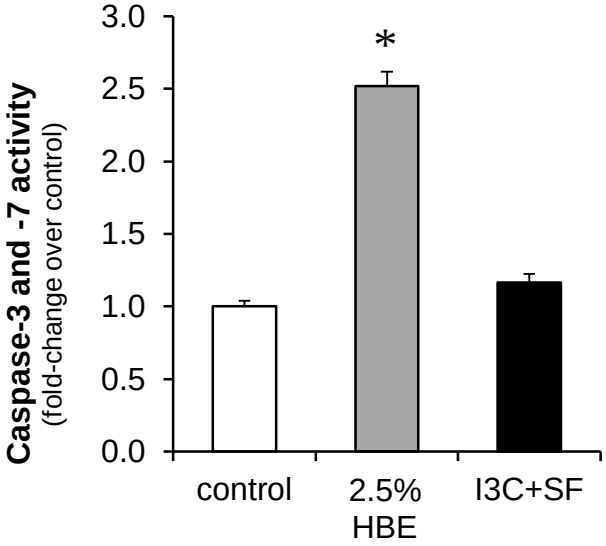


Figure 4.6. Dose-dependent effects of SF, quercetin and kaempferol on NQO1 activity. Murine Hepa-1c1c7 hepatoma cells were exposed for 24 h to (A) purified SF (0.25 - 2 μM), (B) purified quercetin (0.5 - 15 μM), or (C) purified kaempferol (1.25 - 15 μM). Enzyme activity was determined after 24 h exposure. Data, expressed as percent increase over control (untreated cells, 0%) after 24 h treatment, represent mean ± SEM, n=4.

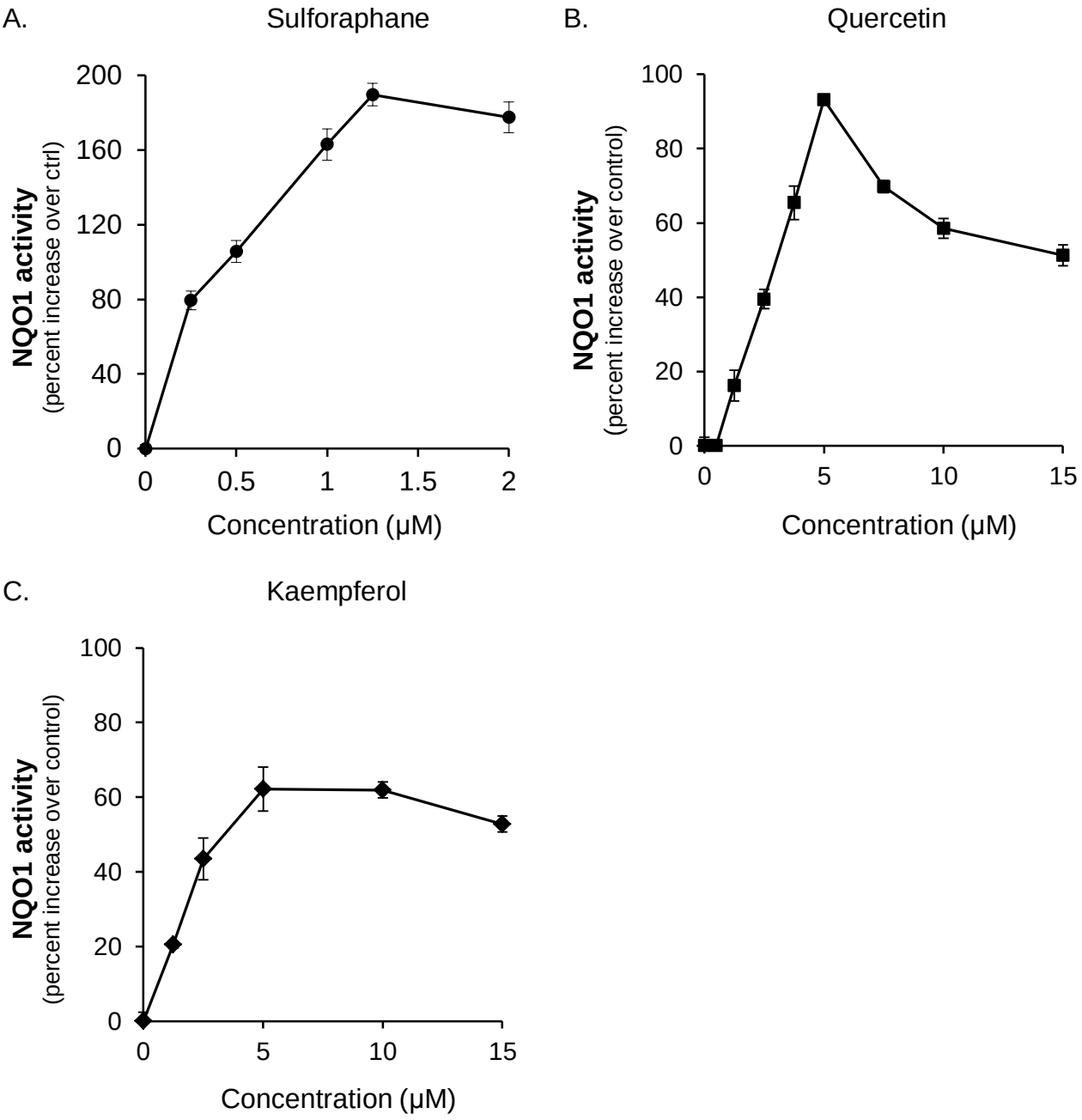
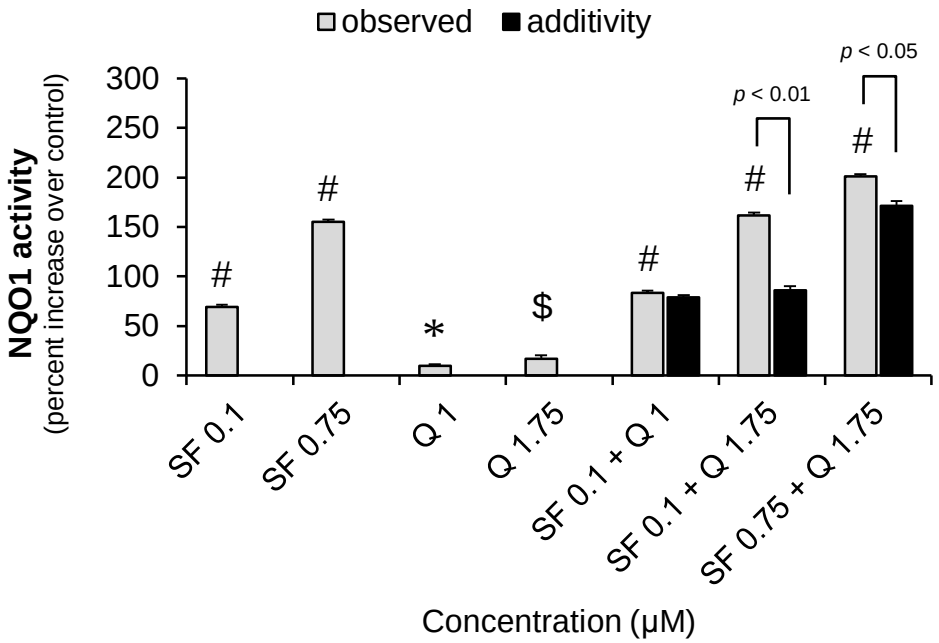


Figure 4.7. Interactive effects among SF, quercetin and kaempferol on NQO1 activity. Murine Hepa-1c1c7 hepatoma cells were exposed for 24 h to (A) combinations of purified SF plus quercetin (Q), (B) purified SF plus kaempferol (K), or (C) purified quercetin plus kaempferol. Enzyme activity was determined after 24 h exposure. Data, expressed as percent increase over control (untreated cells, 0%) after 24 h treatment, represent mean ± SEM, n=4.

A.



B.

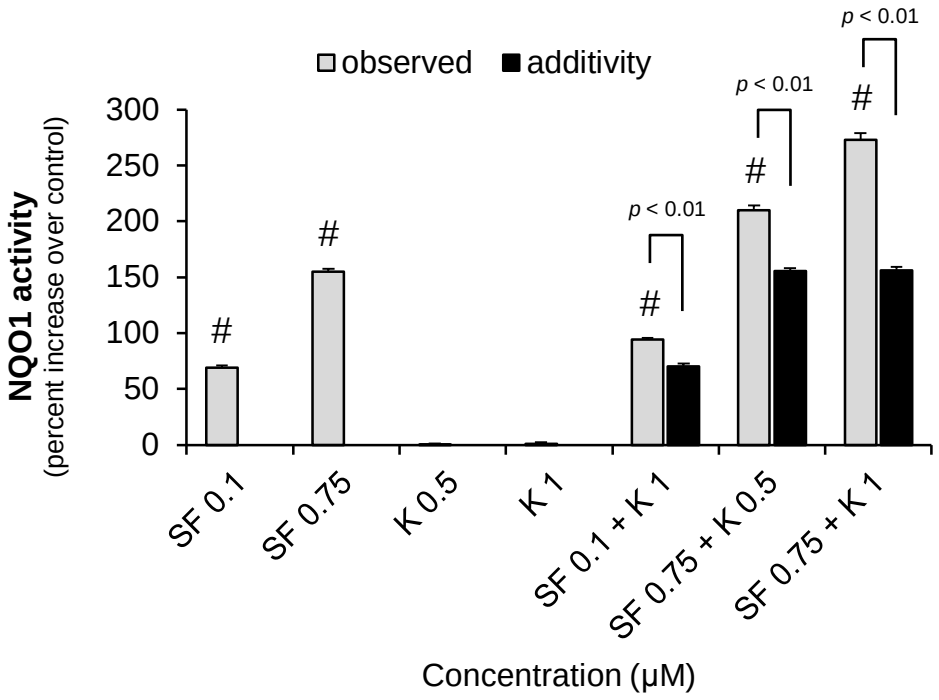
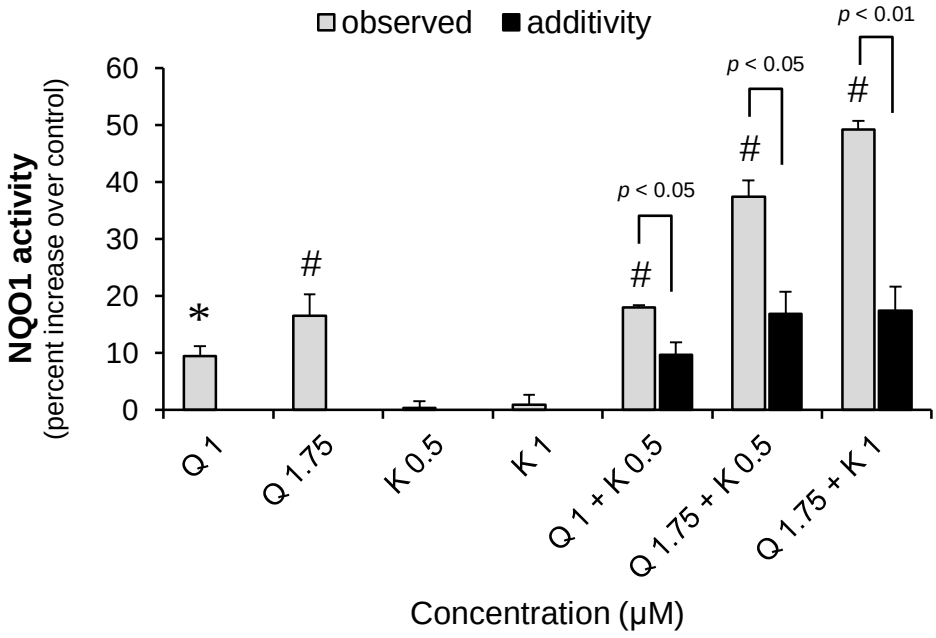


Figure 4.7 (continued)

C.



CHAPTER 5[2]

Selenium enrichment of broccoli alters its bioactivity via changes in the glucosinolate profile

5.1. Introduction

Selenium has gained much attention as a cancer preventive agent since the publication of the Nutritional Prevention of Cancer trial (NPC), which showed that daily supplementation of 200 μg selenium from selenized yeast reduced the incidence of lung, colorectal and prostate cancer in men with a history of either basal cell or squamous cell carcinoma (182). The major selenium species in selenized yeast is selenomethionine (183), which was therefore selected for the more recent Selenium and Vitamin E Cancer Prevention Trial (SELECT). In this large study, daily supplementation of selenomethionine (either alone or in combination with vitamin E) failed to lower the incidence of prostate cancer in relatively healthy men (184). A key reason for this lack of effect may have been the choice of selenium formulation. Although selenomethionine was the primary bioactive component in selenized yeast, other forms of selenium present in the yeast, such as Se-methylselenocysteine (Se-MSC), may have been responsible for the observed reduction in cancer incidence (186).

Crucifers typically contain low amounts of selenium, but have the ability to actively accumulate selenium when grown on selenium-rich soil (191, 196). Analysis of selenium-enriched crucifers has shown that Se-MSC is the major selenium species in these vegetables (183). Multiple animal studies have demonstrated the enhanced

[2] Table 5.1. is adapted with permission from Liu AG, Volker SE, Jeffery EH, Erdman JW. Feeding tomato and broccoli powders enriched with bioactives improves bioactivity markers in rats. J Agric Food Chem. 2009 Aug 26;57(16):7304-10. Copyright 2009 American Chemical Society.

anticarcinogenic potential of selenium-enriched broccoli and other cruciferous vegetables compared to their non-enriched counterparts (194-196). These studies suggest a potential role for Se-MSC and/or its metabolite(s) in cancer prevention.

A possible mechanism for the greater protection against chemically induced carcinogenesis by selenium-enriched broccoli is enhanced upregulation of detoxification enzyme activity (21). However, selenium enrichment has also been shown to increase the glucosinolate content in the plant tissue (21, 204). Whether the observed increase in detoxification enzyme activity is a direct effect of selenium accumulation, or mediated via alterations in the glucosinolate profile of the plant has not been determined.

The purpose of this study was to evaluate the role of selenium in enhanced upregulation of CYP1A and NQO1 activity by selenium-enriched broccoli using the murine Hepa-1c1c7 hepatoma cell line. I hypothesized that selenium-enriched broccoli increases CYP1A and NQO1 activity to a greater extent than low-selenium broccoli. I also hypothesized that addition of Se-MSC, but not selenite, to low-selenium broccoli enhances its bioactivity.

5.2. Methods

Plant material, chemicals and reagents. Standard, low-selenium (0.25 ppm) broccoli florets (*Brassica oleracea* var. Monaco), freeze-dried and ground to a powder, were obtained from FutureCeuticals (Momence, IL); moderate (1.44 ppm) and high-selenium (4.28 ppm) broccoli (*Brassica oleracea* var. Majestic) were grown by Dr. Gary Banuelos at the USDA-ARS research facility in Parlier, CA. Moderate-selenium broccoli was produced via combined treatment with methyl jasmonate and sodium selenate;

high-selenium broccoli was produced by sodium selenate treatment only, all as described previously (21). The glucosinolate and selenium profiles of these broccoli powders are summarized in Table 1. Unless noted otherwise, chemicals and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Cell culture. Murine Hepa-1c1c7 hepatocellular carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Eagle's minimum essential medium with alpha modification, supplemented with 2.2 g/L sodium bicarbonate and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Treatments. Aqueous broccoli extracts were prepared as described previously (Chapter 3) and added to the culture medium at 2.5% final concentration, equivalent to 1.25 mg broccoli powder per mL. Selenite and Se-MSC were dissolved in water, sterile-filtered and stored at -20°C, and added to the broccoli hydrolysis extracts prior to cell treatment. Purified neoglucobrassicin was kindly provided by Dr. M.A. Berhow, USDA-ARS Peoria, IL. Neoglucobrassicin (20 mM) was hydrolyzed in the presence of myrosinase (β-thioglucosidase; E.C. 3.2.1.147, 20 mU/mL) for 24 h at room temperature away from light, sterile-filtered and added to cell culture medium at 0.4% final concentration. All other treatments, except broccoli hydrolysis extracts, were added to the culture medium at 0.1% final concentration.

Enzyme activity. For measuring enzyme activity *in vitro*, Hepa-1c1c7 cells were seeded at 1 x 10^4 cells per well into flat bottom 96-well plates (Corning) and allowed to adhere overnight. Medium was replaced with medium containing treatments, and cells were treated for 24 h. Cellular CYP1A and NQO1 enzyme activity was measured as

described previously (Chapter 3). The activity of CYP1A was measured as ethoxyresorufin *O*-deethylase activity (214); NQO1 activity was measured according to the method of Prochaska and Santamaria (215) with modification (216), both as described previously (Chapter 3).

Statistical analysis. Data were evaluated by one-way analysis of variance using SAS Statistical software (SAS Institute, Cary, NC), and values were considered to be different among treatments at $p < 0.05$ using Fisher's least significant difference procedure.

5.3. Results

Effect of selenium-enrichment on detoxification enzyme activity. The effect of selenium-enrichment on CYP1A and NQO1 activity was determined in murine Hepa-1c1c7 hepatoma cells. Enzyme activity was measured after 24 h treatment.

Low-selenium broccoli hydrolysis extract (2.5%) increased CYP1A activity 14.2-fold compared to control; high-selenium broccoli hydrolysis extract increased enzyme activity 25-fold over control (Fig. 5.1A). NQO1 activity was increased 4.1- and 5.2-fold over control by low- and high-selenium hydrolysis broccoli extracts, respectively (Fig. 5.1B).

Addition of selenium as purified selenite to low-selenium broccoli hydrolysis extract, at concentrations equimolar to high-selenium broccoli, increased CYP1A activity 11.4-fold compared to control, but this increase was not significantly different from the level of enzyme activity observed after treatment with low-selenium broccoli hydrolysis extract alone. Addition of selenium as purified Se-MSC to low-selenium broccoli

hydrolysis extract, at concentrations equimolar to high-selenium broccoli hydrolysis extract, increased CYP1A activity 12.9-fold compared to control. Although this increase in enzyme activity was greater than the one observed after treatment with low-selenium broccoli hydrolysis extract alone, it was still significantly lower than the level of CYP1A activity observed after treatment with high-selenium broccoli hydrolysis extract (Fig. 5.1C).

Addition of selenium, either as purified selenite or Se-MSC, to low-selenium broccoli hydrolysis extract, at concentrations equimolar to high-selenium broccoli hydrolysis extract, increased NQO1 activity ~4.3-fold compared to control, but this increase was not significantly different from the level of enzyme activity observed after treatment with low-selenium broccoli hydrolysis extract alone (Fig. 5.2D).

Moderate-selenium broccoli hydrolysis extract increased CYP1A activity 27.2-fold over control; high-selenium broccoli hydrolysis extract increased enzyme activity 26.2-fold over control (Fig. 5.1E). NQO1 activity was increased 4.1-fold over control by both medium- and high-selenium broccoli hydrolysis extracts (Fig. 5.1F).

Effect of neoglucobrassicin hydrolysis products on CYP1A activity. Prehydrolyzed neoglucobrassicin, at levels that are expected to be found in the high-selenium broccoli hydrolysis extract, increased CYP1A activity in a dose-dependent manner: enzyme activity was increased 3.7-13.7-fold compared to control when 7.5 - 50 μM prehydrolyzed neoglucobrassicin was added (Fig. 5.2).

5.4. Discussion

This study examined the bioactivity (measured as CYP1A and NQO1 activity in Hepa-1c1c7 cells) of broccoli powders with different levels of selenium *in vitro*. In this study, a high-selenium broccoli hydrolysis extract increased CYP1A and NQO1 activity to a greater extent than a low-selenium broccoli hydrolysis extract (Fig. 5.1A, B). Addition of selenium, either as purified selenite or Se-MSC, to the low-selenium broccoli hydrolysis extract had a marginal effect on CYP1A activity (Fig. 5.2C) and no effect on NQO1 activity (Fig. 5.1D). These results suggest that preharvest selenium enrichment of broccoli enhances its bioactivity, whereas postharvest addition of selenium, as either selenite or Se-MSC, to low-selenium broccoli does not.

This study also showed that hydrolysis extracts of broccoli powders with different levels of selenium (1.44 vs. 4.28 ppm), but comparable levels of the indole glucosinolate neoglucobrassicin (13.15 vs. 11.99 μmol/g dry weight), increased CYP1A and NQO1 activity to a similar extent (Fig. 5.1E, F). In addition, hydrolysis products of neoglucobrassicin, at levels expected to be present in the selenium-enriched broccoli powder hydrolysates, increased CYP1A activity. Taken together, these results suggest that the enhanced bioactivity observed for selenium enriched broccoli is not due to a direct effect of selenium on detoxification enzyme activity. Instead, increased levels of neoglucobrassicin in the plant tissue appear to account, in part, for the observed increased bioactivity.

Selenium enrichment has been shown to increase formation of neoglucobrassicin and concomitantly decrease formation of glucobrassicin (21). Since neoglucobrassicin is formed from glucobrassicin via a methylation step (262), it is possible that increased

levels of selenium in the plant tissue alter biosynthesis of glucosinolates to favor formation of neoglucobrassicin from its precursor. The enzyme that catalyzes methylation of glucobrassicin to form neoglucobrassicin is currently unknown.

The neoglucobrassicin hydrolysis product N-methoxyindole-3-carbinol (NI3C) has been shown to be a more efficient inducer of CYP1A activity compared to I3C *in vitro* (220). Therefore, NI3C is proposed to be, in part, responsible for the observed induction of CYP1A by selenium-enriched broccoli. N-methoxyindole-3-carbinol has also been shown to induce cell cycle arrest in human colon cancer cells via upregulation of the cell cycle regulator p21, which may contribute to the enhanced chemoprotective properties of selenium-enriched broccoli (263).

Although selenium-enriched broccoli has been shown to offer enhanced protection against chemically induced cancers *in vivo*, previous reports have cautioned that high levels of selenium uptake may interfere with sulfur uptake and, subsequently, glucosinolate formation. Selenium enrichment has been shown to reduce glucosinolate levels in the plant, but this was at much greater levels of selenium in the plant tissue. Selenium accumulation at 879 µmol/g dry weight resulted in a 43% decrease in aliphatic glucosinolates, while indole glucosinolate levels were not affected (204). However, in another study selenium accumulation decreased levels of both aliphatic and indole glucosinolates (264). In this study, we have shown that selenium enrichment enhanced the bioactivity of broccoli by increasing its glucosinolate content, but the extent of this effect may depend on the amount of selenium applied to the plant, and possibly the genotype of the broccoli (265).

In this study, high-selenium (4.28 ppm) broccoli hydrolysis extract enhanced CYP1A and NQO1 activity by 80% and 25% over low-selenium (0.25 ppm) broccoli hydrolysis extract in Hepa-1c1c7 cells (Fig 5.1A, B). A similar increase in hepatic CYP1A and NQO1 activity was observed in rats fed high -selenium broccoli powder compared to low-selenium broccoli powder (21). This validates the use of Hepa-1c1c7 cells to evaluate the role of selenium-enrichment in enhancement of broccoli bioactivity.

Whether selenium accumulation in the plant also resulted in elevated levels of selenium in culture medium remains unanswered. Glutathione peroxidase assays will be used in future studies to assess selenium bioavailability from the different broccoli powders. Culture medium is routinely found to be selenium deficient, which may inhibit glutathione peroxidase activity (266). A dose-dependent increase in enzyme activity by broccoli hydrolysis extracts will show that, while selenium from the broccoli powders was available and bioactive, it did not affect CYP1A and NQO1 activity.

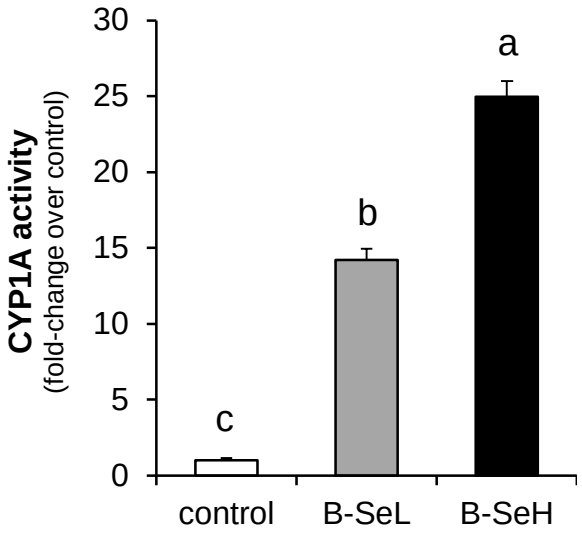
5.5. Tables and figures

Table 5.1. Glucosinolate and selenium profile of broccoli powders. Adapted with permission from Liu AG, Volker SE, Jeffery EH, Erdman JW. Feeding tomato and broccoli powders enriched with bioactives improves bioactivity markers in rats. J Agric Food Chem. 2009 Aug 26;57(16):7304-10. Copyright 2009 American Chemical Society.

	Glucoraphanin (μmol/g dry weight)	Glucobrassicin (μmol/g dry weight)	Neoglucobrassicin (μmol/g dry weight)	Se (ppm)
low-Se	3.05 ± 0.36	2.17 ± 0.06	1.97 ± 0.06	0.25
medium-Se	3.53 ± 0.04	0.70 ± 0.02	13.15 ± 0.33	1.44
high Se	3.36 ± 0.55	0.73 ± 0.05	11.99 ± 0.75	4.28

Figure 5.1. The effect of broccoli selenium-enrichment on detoxification enzyme activity in murine Hepa-1c1c7 hepatoma cells. Cells were exposed for 24 h to broccoli extract (2.5%) with or without selenium as either selenite (sel) or Se-MSC. Enzyme activity was measured after 24 h exposure time. Data, expressed as fold-change over control, represent mean ± SEM, n=4; values that differ are indicated by different letters ($p < 0.05$). B-SeL = low selenium broccoli; B-SeM = medium selenium broccoli; B-SeH = high-selenium broccoli; Se = selenite

A.



B.

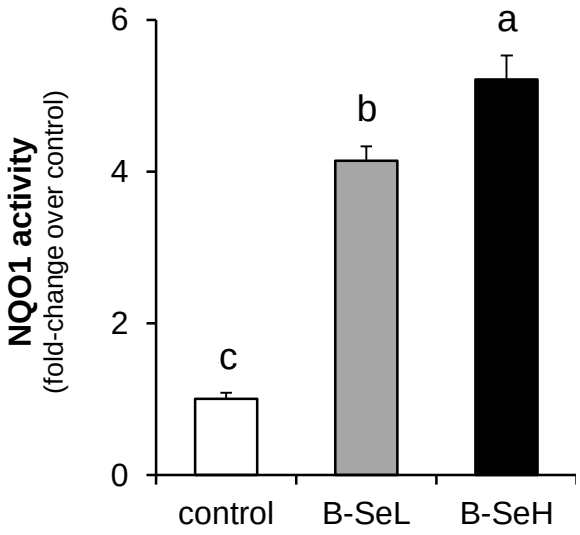
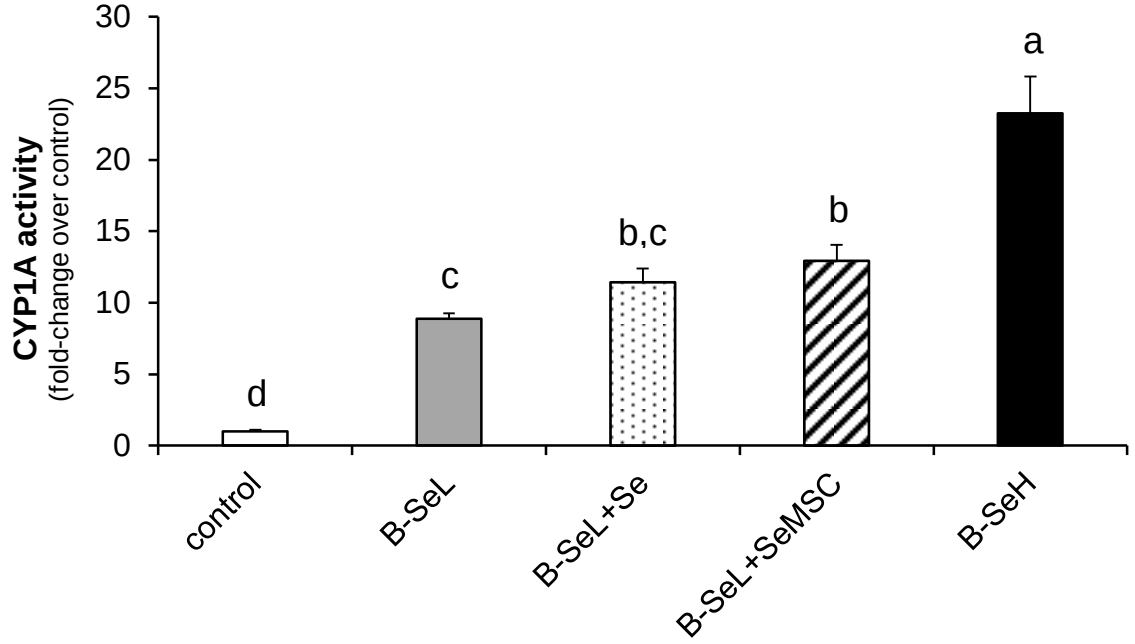


Figure 5.1. (continued)

C.



D.

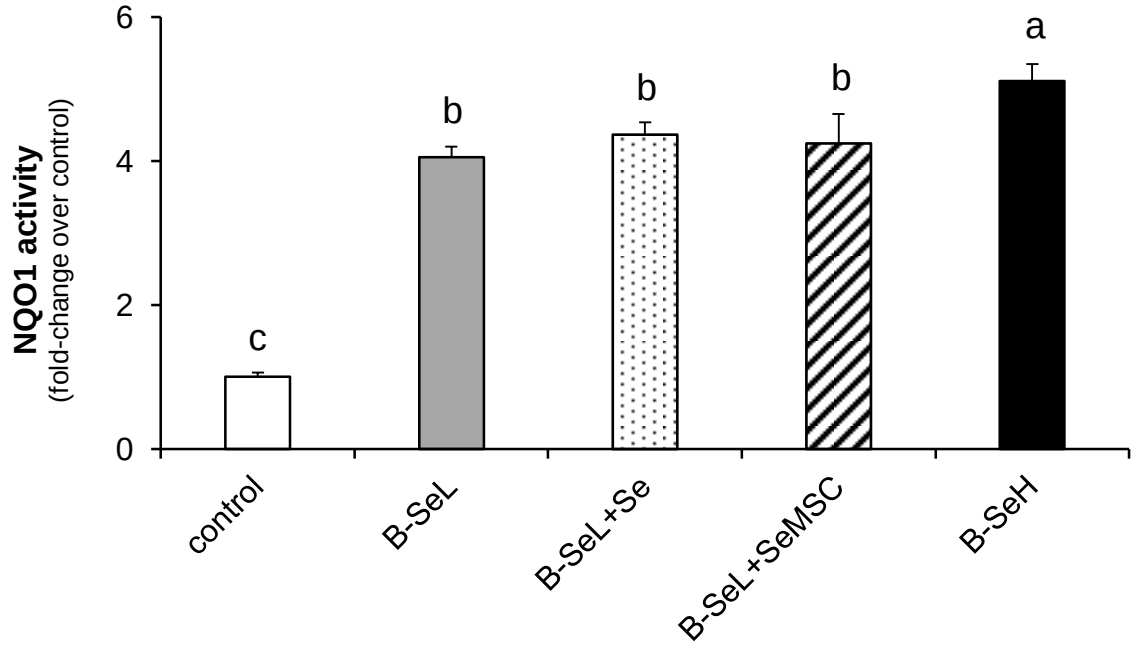
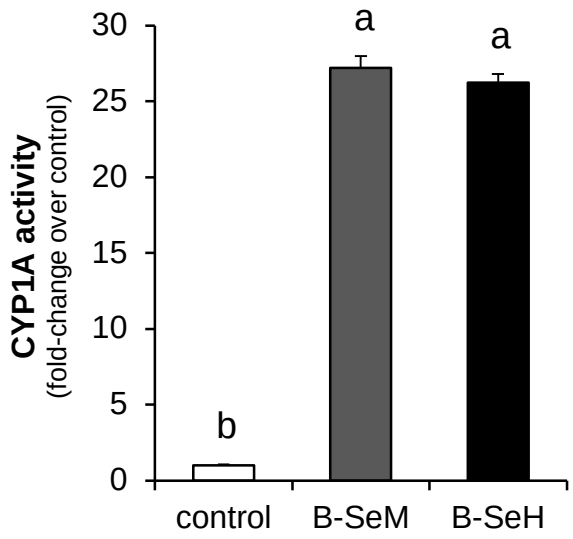


Figure 5.1. (continued)

E.



F.

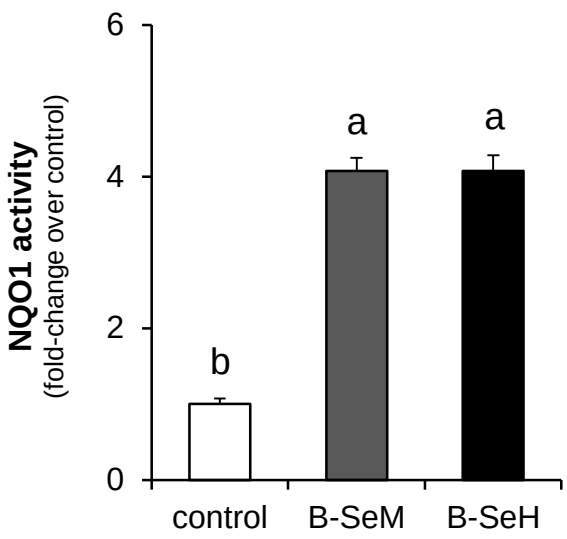
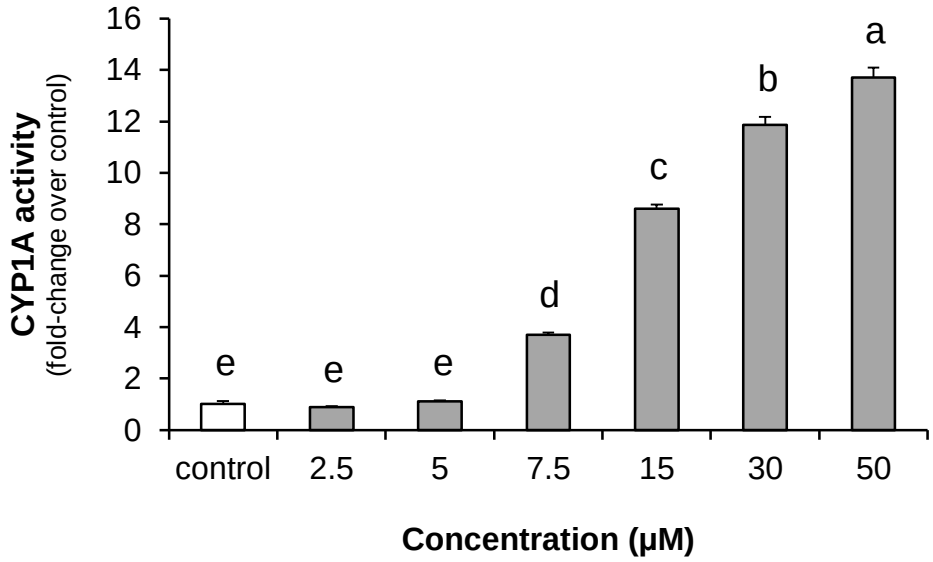


Figure 5.2. The effect of neoglucobrassicin hydrolysis products on detoxification enzyme activity in murine Hepa-1c1c7 hepatoma cells. Cells were exposed for 24 h to increasing amounts of hydrolyzed neoglucobrassicin (2.5 - 50 µM). Enzyme activity was measured after 24 h exposure time. Data, expressed as fold-change over control, represent mean ± SEM, n=4; values that differ are indicated by different letters ($p < 0.05$).



CHAPTER 6

Conclusions and future directions

A healthy lifestyle includes a diet rich in fruits and vegetables. Not only do fruits and vegetables provide vitamins and minerals that are essential for maintaining fundamental functions of the body, they also contain bioactive compounds, which are nonessential components that are associated with reduced risk for chronic diseases, including cancer. Within the different groups of fruits and vegetables, cruciferous vegetables have gained considerable attention for their protective effect against various types of cancer, including those associated with estrogen status. A proposed mechanism for protection against these cancers is alteration of estrogen metabolism. This study showed for the first time that broccoli increased formation of the anticarcinogenic estrogen metabolite 2-methoxyestradiol, which may contribute to the chemoprotective properties of the vegetable.

In Chapter 3, broccoli was not able to inhibit estrogen-induced cancer cell growth, but this may have been due to specific experimental conditions, such as high concentrations of estrogen in the culture medium. Supplementation with the purified glucosinolate breakdown product I3C was shown to increase 2-methoxyestradiol formation in chicken liver after a 7-day feeding trial. Based on this result, a long-term feeding study involving whole broccoli and purified I3C is proposed in the chicken, a preclinical model for ovarian cancer. Outcomes of this study will hopefully provide a better understanding of the impact of broccoli on whole-body estrogen metabolism, and on the development of ovarian cancer and other estrogen-associated cancers.

Epidemiological studies that highlight the health-promoting properties of fruits and vegetables focus on consumption of the whole food. In contrast, many of the plant compounds that are thought to be responsible for this added health benefit are studied *in vitro* in their purified form. While these studies have provided insight into the possible mechanisms underlying the health benefits, they may not accurately reflect the effect of the whole food. Bioactive compounds are often present in the plant as conjugates or (inactive) precursors, which may differ in bioavailability and stability from their purified form. In addition, many bioactive compounds are metabolized extensively in the intestine prior to absorption into the blood stream. Therefore, both the dose and chemical form of a bioactive compound found in the plasma following consumption of the whole food may be very different from those used for *in vitro* studies, which limits the physiological relevance of the study outcomes. Whole foods usually contain multiple bioactive components, whose interactions may result in antagonistic, additive or synergistic effects.

Chapter 4 showed that broccoli as a whole food had a greater effect on multiple study endpoints (induction of detoxification enzyme activity, inhibition of cancer cell growth, induction of caspase activity) compared to a combination of the purified glucosinolate breakdown products I3C plus SF. Chapter 4 also showed interactive effects between SF and other bioactive compounds found in broccoli, the flavonols quercetin and kaempferol, on NQO1 activity. Taken together, these results clearly show that broccoli provides bioactive compounds other than the glucosinolate hydrolysis products. It can also be concluded that these hydrolysis products, as often found in dietary supplements, are not solely responsible for the health benefits of broccoli. More

likely, they are the result of interactive effects between multiple bioactive components present in the plant. Therefore, the added health benefit associated with a diet rich in plant-based foods, may not be achieved through consumption of dietary supplements (which often contain only a single bioactive) alone.

The results of Chapter 4 emphasize the need for research that tries to connect the health benefits of the whole food to the effects observed for the purified compounds. More information is needed regarding the chemical form and concentration of bioactives present in the plasma when consuming a diet that is associated with reduced cancer risk. *In vitro* studies using the physiologically relevant forms and concentrations of these bioactives may help identify compounds that are most likely to have a protective effect in humans, and their underlying mechanisms.

Another approach to bridge the gap between whole foods and purified bioactives is enrichment of foods with specific bioactives, either through agronomic measures or selective breeding. An example is selenium enrichment of crucifers, which have been shown to offer greater protection against chemically-induced carcinogenesis (186, 190, 194-196). Chapter 5 showed that selenium enrichment of broccoli enhanced its chemoprotective potential through alterations in its glucosinolate profile. Selection of different phenotypes may help identify or develop a broccoli with even greater anticarcinogenic properties.

Ultimately, the outcomes of future studies may lead to the development of foods that can provide superior health benefits to entire populations.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin. 2010 Sep-Oct;60(5):277-300.

2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.

3. Doll R, Peto R. The causes of cancer: Quantitative estimates of avoidable risks of cancer in the united states today. J Natl Cancer Inst. 1981 Jun;66(6):1191-308.

4. Kushi LH, Byers T, Doyle C, Bandera EV, McCullough M, McTiernan A, et al. American cancer society guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. CA Cancer J Clin. 2006 Sep-Oct;56(5):254,8:313-4.

5. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: A review. J Am Diet Assoc. 1996 Oct;96(10):1027-39.

6. Sacerdote C, Matullo G, Polidoro S, Gamberini S, Piazza A, Karagas MR, et al. Intake of fruits and vegetables and polymorphisms in DNA repair genes in bladder cancer. Mutagenesis. 2007 Jul;22(4):281-5.

7. Hara M, Hanaoka T, Kobayashi M, Otani T, Adachi HY, Montani A, et al. Cruciferous vegetables, mushrooms, and gastrointestinal cancer risks in a multicenter, hospital-based case-control study in japan. Nutr Cancer. 2003;46(2):138-47.

8. Halkier BA, Gershenzon J. Biology and biochemistry of glucosinolates. Annu Rev Plant Biol. 2006;57:303-33.

9. Cavalieri E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, et al. Catechol estrogen quinones as initiators of breast and other human cancers: Implications for biomarkers of susceptibility and cancer prevention. Biochim Biophys Acta. 2006 Aug;1766(1):63-78.

10. Higdon JV, Delage B, Williams DE, Dashwood RH. Cruciferous vegetables and human cancer risk: Epidemiologic evidence and mechanistic basis. Pharmacol Res. 2007 Mar;55(3):224-36.

11. Im A, Vogel VG, Ahrendt G, Lloyd S, Ragin C, Garte S, et al. Urinary estrogen metabolites in women at high risk for breast cancer. Carcinogenesis. 2009 Sep;30(9):1532-5.

12. Muti P, Bradlow HL, Micheli A, Krogh V, Freudenheim JL, Schunemann HJ, et al. Estrogen metabolism and risk of breast cancer: A prospective study of the 2:16alpha-hydroxyestrone ratio in premenopausal and postmenopausal women. Epidemiology. 2000 Nov;11(6):635-40.

13. Seeger H, Wallwiener D, Kraemer E, Mueck AO. Estradiol metabolites are potent mitogenic substances for human ovarian cancer cells. Eur J Gynaecol Oncol. 2005;26(4):383-5.

14. Barba M, Yang L, Schunemann HJ, Sperati F, Grioni S, Stranges S, et al. Urinary estrogen metabolites and prostate cancer: A case-control study and meta-analysis. J Exp Clin Cancer Res. 2009 Oct 8;28:135.

15. Convert O, Van Aerden C, Debrauwer L, Rathahao E, Molines H, Fournier F, et al. Reactions of estradiol-2,3-quinone with deoxyribonucleosides: Possible insights in the reactivity of estrogen quinones with DNA. Chem Res Toxicol. 2002 May;15(5):754-64.

16. Cavalieri EL, Kumar S, Todorovic R, Higginbotham S, Badawi AF, Rogan EG. Imbalance of estrogen homeostasis in kidney and liver of hamsters treated with estradiol: Implications for estrogen-induced initiation of renal tumors. Chem Res Toxicol. 2001 Aug;14(8):1041-50.

17. Melendez-Colon VJ, Luch A, Seidel A, Baird WM. Cancer initiation by polycyclic aromatic hydrocarbons results from formation of stable DNA adducts rather than apurinic sites. Carcinogenesis. 1999 Oct;20(10):1885-91.

18. Ball P, Knuppen R, Breuer H. Purification and properties of a catechol-o-methyltransferase of human liver. Eur J Biochem. 1971 Aug 25;21(4):517-25.

19. James J, Murry DJ, Treston AM, Storniolo AM, Sledge GW, Sidor C, et al. Phase I safety, pharmacokinetic and pharmacodynamic studies of 2-methoxyestradiol alone or in combination with docetaxel in patients with locally recurrent or metastatic breast cancer. Invest New Drugs. 2007 Feb;25(1):41-8.

20. Sweeney C, Liu G, Yiannoutsos C, Kolesar J, Horvath D, Staab MJ, et al. A phase II multicenter, randomized, double-blind, safety trial assessing the pharmacokinetics, pharmacodynamics, and efficacy of oral 2-methoxyestradiol capsules in hormone-refractory prostate cancer. Clin Cancer Res. 2005 Sep 15;11(18):6625-33.

21. Liu AG, Volker SE, Jeffery EH, Erdman JW. Feeding tomato and broccoli powders enriched with bioactives improves bioactivity markers in rats. J Agric Food Chem. 2009 Aug 26;57(16):7304-10.

22. Tsuchiya Y, Nakajima M, Yokoi T. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. Cancer Lett. 2005 Sep 28;227(2):115-24.

23. Eliassen AH, Hankinson SE. Endogenous hormone levels and risk of breast, endometrial and ovarian cancers: Prospective studies. Adv Exp Med Biol. 2008;630:148-65.

24. Russo J, Russo IH. The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol. 2006 Dec;102(1-5):89-96.

25. Leav I, Merk FB, Kwan PW, Ho SM. Androgen-supported estrogen-enhanced epithelial proliferation in the prostates of intact noble rats. Prostate. 1989;15(1):23-40.

26. Bosland MC, Ford H, Horton L. Induction at high incidence of ductal prostate adenocarcinomas in NBL/Cr and sprague-dawley hsd:SD rats treated with a combination of testosterone and estradiol-17 beta or diethylstilbestrol. Carcinogenesis. 1995 Jun;16(6):1311-7.

27. McPherson K, Steel CM, Dixon JM. ABC of breast diseases. breast cancer-epidemiology, risk factors, and genetics. BMJ. 2000 Sep 9;321(7261):624-8.

28. SEER cancer statistics review 1975-2008 [Internet].; cited 2/26/2012]. Available from: http://seer.cancer.gov/csr/1975_2008/index.html.

29. Castagnetta LA, Miceli MD, Sorci CM, Pfeffer U, Farruggio R, Oliveri G, et al. Growth of LNCaP human prostate cancer cells is stimulated by estradiol via its own receptor. Endocrinology. 1995 May;136(5):2309-19.

30. Ross R, Bernstein L, Judd H, Hanisch R, Pike M, Henderson B. Serum testosterone levels in healthy young black and white men. J Natl Cancer Inst. 1986 Jan;76(1):45-8.

31. Shibata Y, Ito K, Suzuki K, Nakano K, Fukabori Y, Suzuki R, et al. Changes in the endocrine environment of the human prostate transition zone with aging: Simultaneous quantitative analysis of prostatic sex steroids and comparison with human prostatic histological composition. Prostate. 2000 Jan;42(1):45-55.

32. Witschi H. A short history of lung cancer. Toxicol Sci. 2001 Nov;64(1):4-6.

33. Parkin DM, Ferlay J, Curado MP, Bray F, Edwards B, Shin HR, et al. Fifty years of cancer incidence: CI5 I-IX. Int J Cancer. 2010 Dec 15;127(12):2918-27.

34. Ziegler RG, Hoover RN, Pike MC, Hildesheim A, Nomura AM, West DW, et al. Migration patterns and breast cancer risk in asian-american women. J Natl Cancer Inst. 1993 Nov 17;85(22):1819-27.

35. Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM. Cancers of the prostate and breast among japanese and white immigrants in los angeles county Br J Cancer. 1991 Jun;63(6):963-6.

36. Deapen D, Liu L, Perkins C, Bernstein L, Ross RK. Rapidly rising breast cancer incidence rates among asian-american women Int J Cancer. 2002 Jun 10;99(5):747-50.

37. World Cancer Research Fund / American Institute for Cancer Research. Food, nutrition, physical activity and the prevention of cancer: A global perspective. Washington DC: AICR; 2007.

38. Liu RH. Potential synergy of phytochemicals in cancer prevention: Mechanism of action J Nutr. 2004 Dec;134(12 Suppl):3479S-85S.

39. Rattan RS. Mechanism of action of insecticidal secondary metabolites of plant origin Crop Protection. 2010;29(9):913-20.

40. Tan AC, Konczak I, Sze DM, Ramzan I. Molecular pathways for cancer chemoprevention by dietary phytochemicals. Nutr Cancer. 2011;63(4):495-505.

41. Shu L, Cheung KL, Khor TO, Chen C, Kong AN. Phytochemicals: Cancer chemoprevention and suppression of tumor onset and metastasis. Cancer Metastasis Rev. 2010 Sep;29(3):483-502.

42. Pérez-Balibrea S, Moreno DA, García-Viguera C. Genotypic effects on the phytochemical quality of seeds and sprouts from commercial broccoli cultivars. Food Chem. 2011 3/15;125(2):348-54.

43. Murch S, Peiris S, Shi W, Zobayed SMA, Saxena P. Genetic diversity in seed populations of *echinacea purpurea* controls the capacity for regeneration, route of morphogenesis and phytochemical composition. Plant Cell Reports. 2006;25(6):522-32.

44. Bjorkman M, Klingen I, Birch AN, Bones AM, Bruce TJ, Johansen TJ, et al. Phytochemicals of brassicaceae in plant protection and human health--influences of climate, environment and agronomic practice. Phytochemistry. 2011 May;72(7):538-56.

45. Brudzynski K, Kim L. Storage-induced chemical changes in active components of honey de-regulate its antibacterial activity. Food Chem. 2011 6/1;126(3):1155-63.

46. Cermak R, Durazzo A, Maiani G, Bohm V, Kammerer DR, Carle R, et al. The influence of postharvest processing and storage of foodstuffs on the bioavailability of flavonoids and phenolic acids. Mol Nutr Food Res. 2009 Sep;53 Suppl 2:S184-93.

47. Rodriguez-Amaya DB. Food carotenoids: Analysis, composition and alterations during storage and processing of foods. Forum Nutr. 2003;56:35-7.

48. Zhang JJ, Ji R, Hu YQ, Chen JC, Ye XQ. Effect of three cooking methods on nutrient components and antioxidant capacities of bamboo shoot (phyllostachys praecox C.D. chu et C.S. chao). J Zhejiang Univ Sci B. 2011 Sep;12(9):752-9.

49. Martínez-Ballesta MC, López-Pérez L, Hernández M, López-Berenguer C, Fernández-García N, Carvajal M. Agricultural practices for enhanced human health. Phytochemistry Reviews. 2008;7(2):251-60.

50. Coughlin SS. Recall bias in epidemiologic studies. J Clin Epidemiol. 1990;43(1):87-91.

51. Juge N, Mithen RF, Traka M. Molecular basis for chemoprevention by sulforaphane: A comprehensive review. Cell Mol Life Sci. 2007 May;64(9):1105-27.

52. Willett WC. Fruits, vegetables, and cancer prevention: Turmoil in the produce section. J Natl Cancer Inst. 2010 Apr 21;102(8):510-1.

53. Verhoeven DT, Goldbohm RA, van Poppel G, Verhagen H, van den Brandt PA. Epidemiological studies on brassica vegetables and cancer risk. Cancer Epidemiol Biomarkers Prev. 1996 Sep;5(9):733-48.

54. Pan SY, Ugnat AM, Mao Y, Wen SW, Johnson KC, Canadian Cancer Registries Epidemiology Research Group. A case-control study of diet and the risk of ovarian cancer. Cancer Epidemiol Biomarkers Prev. 2004 Sep;13(9):1521-7.

55. Bandera EV, Kushi LH, Moore DF, Gifkins DM, McCullough ML. Fruits and vegetables and endometrial cancer risk: A systematic literature review and meta-analysis. Nutr Cancer. 2007;58(1):6-21.

56. Ambrosini GL, de Klerk NH, Fritschi L, Mackerras D, Musk B. Fruit, vegetable, vitamin A intakes, and prostate cancer risk. Prostate Cancer Prostatic Dis. 2008;11(1):61-6.

57. American institute for cancer research (AICR): Broccoli & cruciferous vegetables [Internet]; cited 3/21/2012]. Available from: http://www.aicr.org/foods-that-fight-cancer/broccoli-cruciferous.html.

58. Rask L, Andréasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J. Myrosinase: Gene family evolution and herbivore defense in brassicaceae. Plant Mol Biol. 2000;42(1):93-113.

59. Lund E. Non-nutritive bioactive constituents of plants: Dietary sources and health benefits of glucosinolates Int J Vitam Nutr Res. 2003 Mar;73(2):135-43.

60. Grob K, Matile PH. Vacuolar location of glucosinolates in horseradish root cells. Plant Science Letters. 1979 4;14(4):327-35.

61. Vasanthi HR, Mukherjee S, Das DK. Potential health benefits of broccoli- a chemico-biological overview. Mini Rev Med Chem. 2009 Jun;9(6):749-59.

62. Kushad MM, Brown AF, Kurilich AC, Juvik JA, Klein BP, Wallig MA, et al. Variation of glucosinolates in vegetable crops of brassica oleracea. J Agric Food Chem. 1999 Apr;47(4):1541-8.

63. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. Proc Natl Acad Sci U S A. 1992 Mar 15;89(6):2399-403.

64. Jana S, Mandlekar S. Role of phase II drug metabolizing enzymes in cancer chemoprevention. Curr Drug Metab. 2009 Jul;10(6):595-616.

65. Villeneuve NF, Lau A, Zhang DD. Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: An insight into cullin-ring ubiquitin ligases. Antioxid Redox Signal. 2010 Dec 1;13(11):1699-712.

66. Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. Genes Cells. 2011 Feb;16(2):123-40.

67. Jakubikova J, Sedlak J, Bod'o J, Bao Y. Effect of isothiocyanates on nuclear accumulation of NF-kappaB, Nrf2, and thioredoxin in caco-2 cells J Agric Food Chem. 2006 Mar 8;54(5):1656-62.

68. Jeong WS, Keum YS, Chen C, Jain MR, Shen G, Kim JH, et al. Differential expression and stability of endogenous nuclear factor E2-related factor 2 (Nrf2) by natural chemopreventive compounds in HepG2 human hepatoma cells J Biochem Mol Biol. 2005 Mar 31;38(2):167-76.

69. Hong F, Freeman ML, Liebler DC. Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. Chem Res Toxicol. 2005 Dec;18(12):1917-26.

70. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants Proc Natl Acad Sci U S A. 2002 Sep 3;99(18):11908-13.

71. McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, et al. The cap ‘n’ collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. Cancer Res. 2001;61(8):3299-307.

72. Loub WD, Wattenberg LW, Davis DW. Aryl hydrocarbon hydroxylase induction in rat tissues by naturally occurring indoles of cruciferous plants. J Natl Cancer Inst. 1975 Apr;54(4):985-8.

73. Nebert DW, Dalton TP. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis Nat Rev Cancer. 2006 Dec;6(12):947-60.

74. Boobis AR, Edwards RJ, Adams DA, Davies DS. Dissecting the function of cytochrome P450 Br J Clin Pharmacol. 1996 Jul;42(1):81-9.

75. Safe S. Molecular biology of the ah receptor and its role in carcinogenesis. Toxicol Lett. 2001 Mar 31;120(1-3):1-7.

76. Kohle C, Bock KW. Coordinate regulation of phase I and II xenobiotic metabolisms by the ah receptor and Nrf2. Biochem Pharmacol. 2007 Jun 15;73(12):1853-62.

77. Weng JR, Tsai CH, Kulp SK, Chen CS. Indole-3-carbinol as a chemopreventive and anti-cancer agent. Cancer Lett. 2008 Apr 18;262(2):153-63.

78. Takahashi N, Dashwood RH, Bjeldanes LF, Williams DE, Bailey GS. Mechanisms of indole-3-carbinol (I3C) anticarcinogenesis: Inhibition of aflatoxin B1-DNA adduction and mutagenesis by I3C acid condensation products Food Chem Toxicol. 1995 Oct;33(10):851-7.

79. Nixon JE, Hendricks JD, Pawlowski NE, Pereira CB, Sinnhuber RO, Bailey GS. Inhibition of aflatoxin B1 carcinogenesis in rainbow trout by flavone and indole compounds Carcinogenesis. 1984 May;5(5):615-9.

80. Schut HA, Cummings DA, Smale MH, Josyula S, Friesen MD. DNA adducts of heterocyclic amines: Formation, removal and inhibition by dietary components Mutat Res. 1997 May 12;376(1-2):185-94.

81. Wattenberg LW, Loub WD. Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles Cancer Res. 1978 May;38(5):1410-3.

82. Arif JM, Gairola CG, Kelloff GJ, Lubet RA, Gupta RC. Inhibition of cigarette smoke-related DNA adducts in rat tissues by indole-3-carbinol Mutat Res. 2000 Jul 20;452(1):11-8.

83. Harris KE, Jeffery EH. Sulforaphane and erucin increase MRP1 and MRP2 in human carcinoma cell lines. J Nutr Biochem. 2008 Apr;19(4):246-54.

84. Navarro SL, Li F, Lampe JW. Mechanisms of action of isothiocyanates in cancer chemoprevention: An update. Food Funct. 2011 Oct;2(10):579-87.

85. Aggarwal BB, Ichikawa H. Molecular targets and anticancer potential of indole-3-carbinol and its derivatives. Cell Cycle. 2005 Sep;4(9):1201-15.

86. Clarke JD, Hsu A, Yu Z, Dashwood RH, Ho E. Differential effects of sulforaphane on histone deacetylases, cell cycle arrest and apoptosis in normal prostate cells versus hyperplastic and cancerous prostate cells Mol Nutr Food Res. 2011 Jul;55(7):999-1009.

87. Kandala PK, Srivastava SK. Activation of checkpoint kinase 2 by 3,3'-diindolylmethane is required for causing G2/M cell cycle arrest in human ovarian cancer cells. Mol Pharmacol. 2010 Aug;78(2):297-309.

88. Chinni SR, Li Y, Upadhyay S, Koppolu PK, Sarkar FH. Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene. 2001 May 24;20(23):2927-36.

89. Shankar S, Ganapathy S, Srivastava RK. Sulforaphane enhances the therapeutic potential of TRAIL in prostate cancer orthotopic model through regulation of apoptosis, metastasis, and angiogenesis. Clin Cancer Res. 2008 Nov 1;14(21):6855-66.

90. Rahman KM, Ali S, Aboukameel A, Sarkar SH, Wang Z, Philip PA, et al. Inactivation of NF-kappaB by 3,3'-diindolylmethane contributes to increased apoptosis induced by chemotherapeutic agent in breast cancer cells. Mol Cancer Ther. 2007 Oct;6(10):2757-65.

91. Chinnakannu K, Chen D, Li Y, Wang Z, Dou QP, Reddy GP, et al. Cell cycle-dependent effects of 3,3'-diindolylmethane on proliferation and apoptosis of prostate cancer cells. J Cell Physiol. 2009 Apr;219(1):94-9.

92. Jeon KI, Rih JK, Kim HJ, Lee YJ, Cho CH, Goldberg ID, et al. Pretreatment of indole-3-carbinol augments TRAIL-induced apoptosis in a prostate cancer cell line, LNCaP. FEBS Lett. 2003 Jun 5;544(1-3):246-51.

93. Taylor-Harding B, Agadjanian H, Nassanian H, Kwon S, Guo X, Miller C, et al. Indole-3-carbinol synergistically sensitises ovarian cancer cells to bortezomib treatment Br J Cancer. 2012 Jan 17;106(2):333-43.

94. Myzak MC, Karplus PA, Chung FL, Dashwood RH. A novel mechanism of chemoprotection by sulforaphane: Inhibition of histone deacetylase. Cancer Res. 2004 Aug 15;64(16):5767-74.

95. Meeran SM, Patel SN, Tollefsbol TO. Sulforaphane causes epigenetic repression of hTERT expression in human breast cancer cell lines. PLoS One. 2010 Jul 6;5(7):e11457.

96. Jones PA. Epigenetics in carcinogenesis and cancer prevention. Ann N Y Acad Sci. 2003 Mar;983:213-9.

97. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer Carcinogenesis. 2010 Jan;31(1):27-36.

98. Myzak MC, Hardin K, Wang R, Dashwood RH, Ho E. Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. Carcinogenesis. 2006 Apr;27(4):811-9.

99. Hsu A, Wong CP, Yu Z, Williams DE, Dashwood RH, Ho E. Promoter de-methylation of cyclin D2 by sulforaphane in prostate cancer cells. Clin Epigenetics. 2011;3:3.

100. Long RS, Simmons EE. The liver and estrogen metabolism; report of cases. AMA Arch Intern Med. 1951 Dec;88(6):762-9.

101. Badawi AF, Cavalieri EL, Rogan EG. Role of human cytochrome P450 1A1, 1A2, 1B1, and 3A4 in the 2-, 4-, and 16alpha-hydroxylation of 17beta-estradiol. Metabolism. 2001 Sep;50(9):1001-3.

102. Zahid M, Kohli E, Saeed M, Rogan E, Cavalieri E. The greater reactivity of estradiol-3,4-quinone vs estradiol-2,3-quinone with DNA in the formation of depurinating adducts: Implications for tumor-initiating activity. Chem Res Toxicol. 2006 Jan;19(1):164-72.

103. Gaikwad NW, Rogan EG, Cavalieri EL. Evidence from ESI-MS for NQO1-catalyzed reduction of estrogen ortho-quinones. Free Radic Biol Med. 2007 Nov 1;43(9):1289-98.

104. Van Zijl C, Lottering ML, Steffens F, Joubert A. In vitro effects of 2-methoxyestradiol on MCF-12A and MCF-7 cell growth, morphology and mitotic spindle formation. Cell Biochem Funct. 2008 Sep-Oct;26(5):632-42.

105. Bu S, Blaukat A, Fu X, Heldin NE, Landstrom M. Mechanisms for 2-methoxyestradiol-induced apoptosis of prostate cancer cells. FEBS Lett. 2002 Nov 6;531(2):141-51.

106. Kato S, Sadarangani A, Lange S, Delpiano AM, Vargas M, Branes J, et al. 2-methoxyestradiol mediates apoptosis through caspase-dependent and independent mechanisms in ovarian cancer cells but not in normal counterparts. Reprod Sci. 2008 Nov;15(9):878-94.

107. Li L, Bu S, Backstrom T, Landstrom M, Ulmsten U, Fu X. Induction of apoptosis and G2/M arrest by 2-methoxyestradiol in human cervical cancer HeLaS3 cells. Anticancer Res. 2004 Mar-Apr;24(2B):873-80.

108. Kumar AP, Garcia GE, Slaga TJ. 2-methoxyestradiol blocks cell-cycle progression at G(2)/M phase and inhibits growth of human prostate cancer cells. Mol Carcinog. 2001 Jul;31(3):111-24.

109. Qadan LR, Perez-Stable CM, Anderson C, D'Ippolito G, Herron A, Howard GA, et al. 2-methoxyestradiol induces G2/M arrest and apoptosis in prostate cancer. Biochem Biophys Res Commun. 2001 Aug 3;285(5):1259-66.

110. Cicek M, Iwaniec UT, Goblirsch MJ, Vrabel A, Ruan M, Clohisy DR, et al. 2-methoxyestradiol suppresses osteolytic breast cancer tumor progression in vivo. Cancer Res. 2007 Nov 1;67(21):10106-11.

111. Klauber N, Parangi S, Flynn E, Hamel E, D'Amato RJ. Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. Cancer Res. 1997 Jan 1;57(1):81-6.

112. Ganapathy M, Ghosh R, Jianping X, Zhang X, Bedolla R, Schoolfield J, et al. Involvement of FLIP in 2-methoxyestradiol-induced tumor regression in transgenic adenocarcinoma of mouse prostate model. Clin Cancer Res. 2009 Mar 1;15(5):1601-11.

113. Garcia GE, Wisniewski HG, Lucia MS, Arevalo N, Slaga TJ, Kraft SL, et al. 2-methoxyestradiol inhibits prostate tumor development in transgenic adenocarcinoma of mouse prostate: Role of tumor necrosis factor-alpha-stimulated gene 6. Clin Cancer Res. 2006 Feb 1;12(3 Pt 1):980-8.

114. Matei D, Schilder J, Sutton G, Perkins S, Breen T, Quon C, et al. Activity of 2 methoxyestradiol (panzem((R)) NCD) in advanced, platinum-resistant ovarian cancer and primary peritoneal carcinomatosis: A hoosier oncology group trial. Gynecol Oncol. 2009 Oct;115(1):90-6.

115. Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, et al. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF Cancer Cell. 2003 Apr;3(4):363-75.

116. Barchiesi F, Jackson EK, Fingerle J, Gillespie DG, Odermatt B, Dubey RK. 2-methoxyestradiol, an estradiol metabolite, inhibits neointima formation and smooth muscle cell growth via double blockade of the cell cycle Circ Res. 2006 Aug 4;99(3):266-74.

117. Bu SZ, Huang Q, Jiang YM, Min HB, Hou Y, Guo ZY, et al. p38 mitogen-activated protein kinases is required for counteraction of 2-methoxyestradiol to estradiol-stimulated cell proliferation and induction of apoptosis in ovarian carcinoma cells via phosphorylation bcl-2. Apoptosis. 2006 Mar;11(3):413-25.

118. Sakurai T, Kudo M. Signaling pathways governing tumor angiogenesis. Oncology. 2011;81 Suppl 1:24-9.

119. Folkman J. Role of angiogenesis in tumor growth and metastasis Semin Oncol. 2002 Dec;29(6 Suppl 16):15-8.

120. Merriam GR, MacLusky NJ, Picard MK, Naftolin F. Comparative properties of the catechol estrogens, I: Methylation by catechol-O-methyltransferase and binding to cytosol estrogen receptors Steroids. 1980 Jul;36(1):1-11.

121. Hughes RA, Harris T, Altmann E, McAllister D, Vlahos R, Robertson A, et al. 2-methoxyestradiol and analogs as novel antiproliferative agents: Analysis of three-dimensional quantitative structure-activity relationships for DNA synthesis inhibition and estrogen receptor binding Mol Pharmacol. 2002 May;61(5):1053-69.

122. LaVallee TM, Zhan XH, Herbstritt CJ, Kough EC, Green SJ, Pribluda VS. 2-methoxyestradiol inhibits proliferation and induces apoptosis independently of estrogen receptors alpha and beta. Cancer Res. 2002 Jul 1;62(13):3691-7.

123. Pribluda VS, Gubish ER,Jr, Lavallee TM, Treston A, Swartz GM, Green SJ. 2-methoxyestradiol: An endogenous antiangiogenic and antiproliferative drug candidate. Cancer Metastasis Rev. 2000;19(1-2):173-9.

124. Kim YS, Milner JA. Targets for indole-3-carbinol in cancer prevention. J Nutr Biochem. 2005 Feb;16(2):65-73.

125. Kabat GC, O'Leary ES, Gammon MD, Sepkovic DW, Teitelbaum SL, Britton JA, et al. Estrogen metabolism and breast cancer. Epidemiology. 2006 Jan;17(1):80-8.

126. Fowke JH, Qi D, Bradlow HL, Shu XO, Gao YT, Cheng JR, et al. Urinary estrogen metabolites and breast cancer: Differential pattern of risk found with pre- versus post-treatment collection. Steroids. 2003 Jan;68(1):65-72.

127. Moon YJ, Wang X, Morris ME. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism Toxicol In Vitro. 2006 Mar;20(2):187-210.

128. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: Food sources and bioavailability Am J Clin Nutr. 2004 May;79(5):727-47.

129. De Stefani E, Ronco A, Mendilaharsu M, Deneo-Pellegrini H. Diet and risk of cancer of the upper aerodigestive tract--II. nutrients Oral Oncol. 1999 Jan;35(1):22-6.

130. Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, et al. Flavonoid intake and risk of chronic diseases. Am J Clin Nutr. 2002 Sep;76(3):560-8.

131. Garcia-Closas R, Gonzalez CA, Agudo A, Riboli E. Intake of specific carotenoids and flavonoids and the risk of gastric cancer in spain. Cancer Causes Control. 1999 Feb;10(1):71-5.

132. Knekt P, Jarvinen R, Seppanen R, Hellovaara M, Teppo L, Pukkala E, et al. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms Am J Epidemiol. 1997 Aug 1;146(3):223-30.

133. Le Marchand L, Murphy SP, Hankin JH, Wilkens LR, Kolonel LN. Intake of flavonoids and lung cancer J Natl Cancer Inst. 2000 Jan 19;92(2):154-60.

134. Murakami A, Ashida H, Terao J. Multitargeted cancer prevention by quercetin Cancer Lett. 2008 Oct 8;269(2):315-25.

135. Plumb GW, Price KR, Rhodes MJ, Williamson G. Antioxidant properties of the major polyphenolic compounds in broccoli. Free Radic Res. 1997 Oct;27(4):429-35.

136. Williamson G, Plumb GW, Uda Y, Price KR, Rhodes MJ. Dietary quercetin glycosides: Antioxidant activity and induction of the anticarcinogenic phase II marker enzyme quinone reductase in Hepalclc7 cells. Carcinogenesis. 1996 Nov;17(11):2385-7.

137. Aruoma OI. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods Mutat Res. 2003 Feb-Mar;523-524:9-20.

138. Aaby K, Hvattum E, Skrede G. Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: Relationship to antioxidant activity J Agric Food Chem. 2004 Jul 28;52(15):4595-603.

139. Lotito SB, Frei B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon? Free Radic Biol Med. 2006 Dec 15;41(12):1727-46.

140. Hollman PC, Cassidy A, Comte B, Heinonen M, Richelle M, Richling E, et al. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established J Nutr. 2011 May;141(5):989S-1009S.

141. Ciolino HP, Daschner PJ, Yeh GC. Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. Biochem J. 1999 Jun 15;340 (Pt 3)(Pt 3):715-22.

142. Mandal PK. Dioxin: A review of its environmental effects and its aryl hydrocarbon receptor biology J Comp Physiol B. 2005 May;175(4):221-30.

143. Kang ZC, Tsai SJ, Lee H. Quercetin inhibits benzo[a]pyrene-induced DNA adducts in human hep G2 cells by altering cytochrome P-450 1A1 gene expression Nutr Cancer. 1999;35(2):175-9.

144. Galijatovic A, Walle UK, Walle T. Induction of UDP-glucuronosyltransferase by the flavonoids chrysin and quercetin in caco-2 cells Pharm Res. 2000 Jan;17(1):21-6.

145. van der Logt EM, Roelofs HM, Nagengast FM, Peters WH. Induction of rat hepatic and intestinal UDP-glucuronosyltransferases by naturally occurring dietary anticarcinogens Carcinogenesis. 2003 Oct;24(10):1651-6.

146. Barbier O, Belanger A. Inactivation of androgens by UDP-glucuronosyltransferases in the human prostate Best Pract Res Clin Endocrinol Metab. 2008 Apr;22(2):259-70.

147. Gall WE, Zawada G, Mojarrabi B, Tephly TR, Green MD, Coffman BL, et al. Differential glucuronidation of bile acids, androgens and estrogens by human UGT1A3 and 2B7 J Steroid Biochem Mol Biol. 1999 Jul-Aug;70(1-3):101-8.

148. Valerio LG,Jr, Kepa JK, Pickwell GV, Quattrochi LC. Induction of human NAD(P)H:Quinone oxidoreductase (NQO1) gene expression by the flavonol quercetin. Toxicol Lett. 2001 Feb 3;119(1):49-57.

149. Uda Y, Price KR, Williamson G, Rhodes MJ. Induction of the anticarcinogenic marker enzyme, quinone reductase, in murine hepatoma cells in vitro by flavonoids. Cancer Lett. 1997 Dec 9;120(2):213-6.

150. Ramos S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention J Nutr Biochem. 2007 Jul;18(7):427-42.

151. Choi JA, Kim JY, Lee JY, Kang CM, Kwon HJ, Yoo YD, et al. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin Int J Oncol. 2001 Oct;19(4):837-44.

152. Seo HS, Ju JH, Jang K, Shin I. Induction of apoptotic cell death by phytoestrogens by up-regulating the levels of phospho-p53 and p21 in normal and malignant estrogen receptor alpha-negative breast cells Nutr Res. 2011 Feb;31(2):139-46.

153. Vidya Priyadarsini R, Senthil Murugan R, Maitreyi S, Ramalingam K, Karunagaran D, Nagini S. The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF-kappaB inhibition Eur J Pharmacol. 2010 Dec 15;649(1-3):84-91.

154. Luo H, Rankin GO, Liu L, Daddysman MK, Jiang BH, Chen YC. Kaempferol inhibits angiogenesis and VEGF expression through both HIF dependent and independent pathways in human ovarian cancer cells. Nutr Cancer. 2009;61(4):554-63.

155. Vijayababu MR, Kanagaraj P, Arunkumar A, Ilangovan R, Aruldhas MM, Arunakaran J. Quercetin-induced growth inhibition and cell death in prostatic carcinoma cells (PC-3) are associated with increase in p21 and hypophosphorylated retinoblastoma proteins expression J Cancer Res Clin Oncol. 2005 Nov;131(11):765-71.

156. Lee DH, Szczepanski M, Lee YJ. Role of bax in quercetin-induced apoptosis in human prostate cancer cells Biochem Pharmacol. 2008 Jun 15;75(12):2345-55.

157. Kim YH, Lee YJ. TRAIL apoptosis is enhanced by quercetin through akt dephosphorylation J Cell Biochem. 2007 Mar 1;100(4):998-1009.

158. Luo H, Rankin GO, Li Z, Depriest L, Chen YC. Kaempferol induces apoptosis in ovarian cancer cells through activating p53 in the intrinsic pathway Food Chem. 2011 Sep 15;128(2):513-9.

159. Chien SY, Wu YC, Chung JG, Yang JS, Lu HF, Tsou MF, et al. Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells Hum Exp Toxicol. 2009 Aug;28(8):493-503.

160. Baguley BC. Multidrug resistance in cancer Methods Mol Biol. 2010;596:1-14.

161. Merino V, Jimenez-Torres NV, Merino-Sanjuan M. Relevance of multidrug resistance proteins on the clinical efficacy of cancer therapy Curr Drug Deliv. 2004 Jul;1(3):203-12.

162. Li Y, Revalde JL, Reid G, Paxton JW. Interactions of dietary phytochemicals with ABC transporters: Possible implications for drug disposition and multidrug resistance in cancer Drug Metab Rev. 2010 Nov;42(4):590-611.

163. Chen C, Zhou J, Ji C. Quercetin: A potential drug to reverse multidrug resistance Life Sci. 2010 Sep 11;87(11-12):333-8.

164. Navarro G, Sawant RR, Biswas S, Essex S, Tros de Ilarduya C, Torchilin VP. P-glycoprotein silencing with siRNA delivered by DOPE-modified PEI overcomes doxorubicin resistance in breast cancer cells Nanomedicine (Lond). 2012 Jan;7(1):65-78.

165. Gouaze V, Liu YY, Prickett CS, Yu JY, Giuliano AE, Cabot MC. Glucosylceramide synthase blockade down-regulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs Cancer Res. 2005 May 1;65(9):3861-7.

166. O'Neill AJ, Prencipe M, Dowling C, Fan Y, Mulrane L, Gallagher WM, et al. Characterisation and manipulation of docetaxel resistant prostate cancer cell lines Mol Cancer. 2011 Oct 7;10:126.

167. Tasaki Y, Nakagawa M, Ogata J, Kiue A, Tanimura H, Kuwano M, et al. Reversal by a dihydropyridine derivative of non-P-glycoprotein-mediated multidrug resistance in etoposide-resistant human prostatic cancer cell line J Urol. 1995 Sep;154(3):1210-6.

168. Zhang T, Guan M, Jin HY, Lu Y. Reversal of multidrug resistance by small interfering double-stranded RNAs in ovarian cancer cells Gynecol Oncol. 2005 May;97(2):501-7.

169. Kowalski P, Surowiak P, Lage H. Reversal of different drug-resistant phenotypes by an autocatalytic multitarget multiribozyme directed against the transcripts of the ABC transporters MDR1/P-gp, MRP2, and BCRP Mol Ther. 2005 Apr;11(4):508-22.

170. Limtrakul P, Khantamat O, Pintha K. Inhibition of P-glycoprotein function and expression by kaempferol and quercetin J Chemother. 2005 Feb;17(1):86-95.

171. Gruber BM, Bubko I, Krzyszton-Russjan J, Anuszewska EL. Synergistic action of doxorubicin and sulindac in human cervix carcinoma cells - studies on possible mechanisms Med Sci Monit. 2010 Jan;16(1):BR45-51.

172. Rayman MP. The importance of selenium to human health Lancet. 2000 Jul 15;356(9225):233-41.

173. Fairweather-Tait SJ, Bao Y, Broadley MR, Collings R, Ford D, Hesketh JE, et al. Selenium in human health and disease Antioxid Redox Signal. 2011 Apr 1;14(7):1337-83.

174. Schrauzer GN, White DA, Schneider CJ. Cancer mortality correlation studies--III: Statistical associations with dietary selenium intakes. Bioinorg Chem. 1977;7(1):23-31.

175. Shu-Yu Yu, Ya-Jun Chu, Xin-Lan Gong. Regional variation of cancer mortality incidence and its relation to selenium levels in china. Biol Trace Elem Res. 1985;7(1):21-9.

176. de Rosa V, Erkekoglu P, Forestier A, Favier A, Hincal F, Diamond AM, et al. Low doses of selenium specifically stimulate the repair of oxidative DNA damage in LNCaP prostate cancer cells Free Radic Res. 2012 Feb;46(2):105-16.

177. Wang R, Zhou S, Li S. Cancer therapeutic agents targeting hypoxia-inducible factor-1 Curr Med Chem. 2011;18(21):3168-89.

178. Zhao R, Xiang N, Domann FE, Zhong W. Effects of selenite and genistein on G2/M cell cycle arrest and apoptosis in human prostate cancer cells Nutr Cancer. 2009;61(3):397-407.

179. Lee JT, Lee TJ, Park JW, Kwon TK. Se-methylselenocysteine sensitized TRAIL-mediated apoptosis via down-regulation of bcl-2 expression Int J Oncol. 2009 May;34(5):1455-60.

180. Jiang C, Ganther H, Lu J. Monomethyl selenium--specific inhibition of MMP-2 and VEGF expression: Implications for angiogenic switch regulation Mol Carcinog. 2000 Dec;29(4):236-50.

181. Jiang C, Kim KH, Wang Z, Lu J. Methyl selenium-induced vascular endothelial apoptosis is executed by caspases and principally mediated by p38 MAPK pathway Nutr Cancer. 2004;49(2):174-83.

182. Clark LC, Combs GF,Jr, Turnbull BW, Slate EH, Chalker DK, Chow J, et al. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. nutritional prevention of cancer study group. JAMA. 1996 Dec 25;276(24):1957-63.

183. Rayman MP, Infante HG, Sargent M. Food-chain selenium and human health: Spotlight on speciation. Br J Nutr. 2008 Aug;100(2):238-53.

184. Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, et al. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: The selenium and vitamin E cancer prevention trial (SELECT). JAMA. 2009 Jan 7;301(1):39-51.

185. Klein EA, Thompson IM,Jr, Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, et al. Vitamin E and the risk of prostate cancer: The selenium and vitamin E cancer prevention trial (SELECT) JAMA. 2011 Oct 12;306(14):1549-56.

186. Ip C, Birringer M, Block E, Kotrebai M, Tyson JF, Uden PC, et al. Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. J Agric Food Chem. 2000 Jun;48(6):2062-70.

187. Whanger PD. Selenium and its relationship to cancer: An update. Br J Nutr. 2004 Jan;91(1):11-28.

188. Hatfield DL, Gladyshev VN. The outcome of selenium and vitamin E cancer prevention trial (SELECT) reveals the need for better understanding of selenium biology. Mol Interv. 2009 Feb;9(1):18-21.

189. Thomson CD, Robinson MF, Butler JA, Whanger PD. Long-term supplementation with selenate and selenomethionine: Selenium and glutathione peroxidase (EC 1.11.1.9) in blood components of new zealand women Br J Nutr. 1993 Mar;69(2):577-88.

190. Finley JW, Davis CD, Feng Y. Selenium from high selenium broccoli protects rats from colon cancer. J Nutr. 2000 Sep;130(9):2384-9.

191. Banuelos GS. Irrigation of broccoli and canola with boron- and selenium-laden effluent. J Environ Qual. 2002 Nov-Dec;31(6):1802-8.

192. Ellis DR, Salt DE. Plants, selenium and human health Curr Opin Plant Biol. 2003 Jun;6(3):273-9.

193. Keck AS, Finley JW. Cruciferous vegetables: Cancer protective mechanisms of glucosinolate hydrolysis products and selenium. Integr Cancer Ther. 2004 Mar;3(1):5-12.

194. Yamanoshita O, Ichihara S, Hama H, Ichihara G, Chiba M, Kamijima M, et al. Chemopreventive effect of selenium-enriched japanese radish sprout against breast cancer induced by 7,12-dimethylbenz[a]anthracene in rats. Tohoku J Exp Med. 2007 Jun;212(2):191-8.

195. Finley JW, Ip C, Lisk DJ, Davis CD, Hintze KJ, Whanger PD. Cancer-protective properties of high-selenium broccoli. J Agric Food Chem. 2001 May;49(5):2679-83.

196. Davis CD, Zeng H, Finley JW. Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice. J Nutr. 2002 Feb;132(2):307-9.

197. Li GX, Lee HJ, Wang Z, Hu H, Liao JD, Watts JC, et al. Superior in vivo inhibitory efficacy of methylseleninic acid against human prostate cancer over selenomethionine or selenite. Carcinogenesis. 2008 May;29(5):1005-12.

198. Hurst R, Elliott RM, Goldson AJ, Fairweather-Tait SJ. Se-methylselenocysteine alters collagen gene and protein expression in human prostate cells. Cancer Lett. 2008 Sep 28;269(1):117-26.

199. Verenich S, Gerk PM. Therapeutic promises of 2-methoxyestradiol and its drug disposition challenges Mol Pharm. 2010 Dec 6;7(6):2030-9.

200. Barua A, Bitterman P, Abramowicz JS, Dirks AL, Bahr JM, Hales DB, et al. Histopathology of ovarian tumors in laying hens: A preclinical model of human ovarian cancer. Int J Gynecol Cancer. 2009 May;19(4):531-9.

201. Arnold JT, Le H, McFann KK, Blackman MR. Comparative effects of DHEA vs. testosterone, dihydrotestosterone, and estradiol on proliferation and gene expression in human LNCaP prostate cancer cells Am J Physiol Endocrinol Metab. 2005 Mar;288(3):E573-84.

202. Hsu JC, Dev A, Wing A, Brew CT, Bjeldanes LF, Firestone GL. Indole-3-carbinol mediated cell cycle arrest of LNCaP human prostate cancer cells requires the induced production of activated p53 tumor suppressor protein. Biochem Pharmacol. 2006 Dec 15;72(12):1714-23.

203. Chuang LT, Moqattash ST, Gretz HF, Nezhat F, Rahaman J, Chiao JW. Sulforaphane induces growth arrest and apoptosis in human ovarian cancer cells. Acta Obstet Gynecol Scand. 2007 Jul 16:1-6.

204. Robbins RJ, Keck AS, Banuelos G, Finley JW. Cultivation conditions and selenium fertilization alter the phenolic profile, glucosinolate, and sulforaphane content of broccoli. J Med Food. 2005 Summer;8(2):204-14.

205. Ferlay J, shin HR, bray F, forman D, mathers C and parkin DM. GLOBOCAN 2008 v1.2, cancer incidence and mortality worldwide: IARC CancerBase no. 10 [internet]. Lyon, France: International agency for research on cancer; 2010. available from: http://globocan.iarc.fr, accessed on 03/04/2012. [Internet].

206. Kirsh VA, Peters U, Mayne ST, Subar AF, Chatterjee N, Johnson CC, et al. Prospective study of fruit and vegetable intake and risk of prostate cancer. J Natl Cancer Inst. 2007 Aug 1;99(15):1200-9.

207. Kolonel LN, Hankin JH, Whittemore AS, Wu AH, Gallagher RP, Wilkens LR, et al. Vegetables, fruits, legumes and prostate cancer: A multiethnic case-control study. Cancer Epidemiol Biomarkers Prev. 2000 Aug;9(8):795-804.

208. Jain MG, Hislop GT, Howe GR, Ghadirian P. Plant foods, antioxidants, and prostate cancer risk: Findings from case-control studies in canada. Nutr Cancer. 1999;34(2):173-84.

209. Zhang CX, Ho SC, Chen YM, Fu JH, Cheng SZ, Lin FY. Greater vegetable and fruit intake is associated with a lower risk of breast cancer among chinese women. Int J Cancer. 2009 Jul 1;125(1):181-8.

210. Ambrosone CB, McCann SE, Freudenheim JL, Marshall JR, Zhang Y, Shields PG. Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. J Nutr. 2004 May;134(5):1134-8.

211. Fowke JH, Chung FL, Jin F, Qi D, Cai Q, Conaway C, et al. Urinary isothiocyanate levels, brassica, and human breast cancer. Cancer Res. 2003 Jul 15;63(14):3980-6.

212. Nagle CM, Purdie DM, Webb PM, Green A, Harvey PW, Bain CJ. Dietary influences on survival after ovarian cancer. Int J Cancer. 2003 Aug 20;106(2):264-9.

213. Mueck AO, Seeger H. 2-methoxyestradiol--biology and mechanism of action Steroids. 2010 Oct;75(10):625-31.

214. Paolini M, Perocco P, Canistro D, Valgimigli L, Pedulli GF, Iori R, et al. Induction of cytochrome P450, generation of oxidative stress and in vitro cell-transforming and DNA-damaging activities by glucoraphanin, the bioprecursor of the chemopreventive agent sulforaphane found in broccoli. Carcinogenesis. 2004 Jan;25(1):61-7.

215. Prochaska HJ, Santamaria AB. Direct measurement of NAD(P)H:Quinone reductase from cells cultured in microtiter wells: A screening assay for anticarcinogenic enzyme inducers. Anal Biochem. 1988 Mar;169(2):328-36.

216. Lai RH, Keck AS, Wallig MA, West LG, Jeffery EH. Evaluation of the safety and bioactivity of purified and semi-purified glucoraphanin. Food Chem Toxicol. 2008 Jan;46(1):195-202.

217. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976 May 7;72:248-54.

218. De Kruif CA, Marsman JW, Venekamp JC, Falke HE, Noordhoek J, Blaauboer BJ, et al. Structure elucidation of acid reaction products of indole-3-carbinol: Detection in vivo and enzyme induction in vitro. Chem Biol Interact. 1991;80(3):303-15.

219. Bryan Hanley A, Parsley KR. Identification of 1-methoxyindolyl-3-methyl isothiocyanate as an indole glucosinolate breakdown product. Phytochemistry. 1990;29(3):769-71.

220. Stephensen PU, Bonnesen C, Schaldach C, Andersen O, Bjeldanes LF, Vang O. N-methoxyindole-3-carbinol is a more efficient inducer of cytochrome P-450 1A1 in cultured cells than indol-3-carbinol. Nutr Cancer. 2000;36(1):112-21.

221. Uno S, Endo K, Ishida Y, Tateno C, Makishima M, Yoshizato K, et al. CYP1A1 and CYP1A2 expression: Comparing 'humanized' mouse lines and wild-type mice; comparing human and mouse hepatoma-derived cell lines. Toxicol Appl Pharmacol. 2009 May 15;237(1):119-26.

222. Kishida T, Beppu M, Nashiki K, Izumi T, Ebihara K. Effect of dietary soy isoflavone aglycones on the urinary 16alpha-to-2-hydroxyestrone ratio in C3H/HeJ mice. Nutr Cancer. 2000;38(2):209-14.

223. Tevaarwerk AJ, Holen KD, Alberti DB, Sidor C, Arnott J, Quon C, et al. Phase I trial of 2-methoxyestradiol NanoCrystal dispersion in advanced solid malignancies. Clin Cancer Res. 2009 Feb 15;15(4):1460-5.

224. Matei D, Schilder J, Sutton G, Perkins S, Breen T, Quon C, et al. Activity of 2 methoxyestradiol (panzem NCD) in advanced, platinum-resistant ovarian cancer and primary peritoneal carcinomatosis: A hoosier oncology group trial. Gynecol Oncol. 2009 Oct;115(1):90-6.

225. Bruce JY, Eickhoff J, Pili R, Logan T, Carducci M, Arnott J, et al. A phase II study of 2-methoxyestradiol nanocrystal colloidal dispersion alone and in combination with sunitinib malate in patients with metastatic renal cell carcinoma progressing on sunitinib malate. Invest New Drugs. 2012 Apr;30(2):794-802.

226. Harrison MR, Hahn NM, Pili R, Oh WK, Hammers H, Sweeney C, et al. A phase II study of 2-methoxyestradiol (2ME2) NanoCrystal(R) dispersion (NCD) in patients with taxane-refractory, metastatic castrate-resistant prostate cancer (CRPC). Invest New Drugs. 2011 Dec;29(6):1465-74.

227. Zhou Q, Gustafson D, Nallapareddy S, Diab S, Leong S, Lewis K, et al. A phase I dose-escalation, safety and pharmacokinetic study of the 2-methoxyestradiol analog ENMD-1198 administered orally to patients with advanced cancer. Invest New Drugs. 2011 Apr;29(2):340-6.

228. Michnovicz JJ, Adlercreutz H, Bradlow HL. Changes in levels of urinary estrogen metabolites after oral indole-3-carbinol treatment in humans. J Natl Cancer Inst. 1997 May 21;89(10):718-23.

229. Ansenberger K, Richards C, Zhuge Y, Barua A, Bahr JM, Luborsky JL, et al. Decreased severity of ovarian cancer and increased survival in hens fed a flaxseed-enriched diet for 1 year. Gynecol Oncol. 2010 May;117(2):341-7.

230. Prochaska HJ, De Long MJ, Talalay P. On the mechanisms of induction of cancer-protective enzymes: A unifying proposal. Proc Natl Acad Sci U S A. 1985 Dec;82(23):8232-6.

231. Prestera T, Holtzclaw WD, Zhang Y, Talalay P. Chemical and molecular regulation of enzymes that detoxify carcinogens. Proc Natl Acad Sci U S A. 1993 Apr 1;90(7):2965-9.

232. Keck AS, Finley JW. Aqueous extracts of selenium-fertilized broccoli increase selenoprotein activity and inhibit DNA single-strand breaks, but decrease the activity of quinone reductase in hepa 1c1c7 cells. Food Chem Toxicol. 2006 May;44(5):695-703.

233. Gessner PK. Isobolographic analysis of interactions: An update on applications and utility. Toxicology. 1995 Dec 28;105(2-3):161-79.

234. Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, Talalay P. Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: Pharmacokinetics of broccoli sprout isothiocyanates in humans. Clin Chim Acta. 2002 Feb;316(1-2):43-53.

235. Gasper AV, Al-Janobi A, Smith JA, Bacon JR, Fortun P, Atherton C, et al. Glutathione S-transferase M1 polymorphism and metabolism of sulforaphane from standard and high-glucosinolate broccoli. Am J Clin Nutr. 2005 Dec;82(6):1283-91.

236. Cramer JM, Teran-Garcia M, Jeffery EH. Enhancing sulforaphane absorption and excretion in healthy men through the combined consumption of fresh broccoli sprouts and a glucoraphanin-rich powder. Br J Nutr. 2011 Sep 13:1-6.

237. Chiao JW, Chung FL, Kancherla R, Ahmed T, Mittelman A, Conaway CC. Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. Int J Oncol. 2002 Mar;20(3):631-6.

238. Raj MH, Abd Elmageed ZY, Zhou J, Gaur RL, Nguyen L, Azam GA, et al. Synergistic action of dietary phyto-antioxidants on survival and proliferation of ovarian cancer cells. Gynecol Oncol. 2008 Sep;110(3):432-8.

239. Ola MS, Nawaz M, Ahsan H. Role of bcl-2 family proteins and caspases in the regulation of apoptosis Mol Cell Biochem. 2011 May;351(1-2):41-58.

240. Nioi P, Hayes JD. Contribution of NAD(P)H:Quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors Mutat Res. 2004 Nov 2;555(1-2):149-71.

241. McWalter GK, Higgins LG, McLellan LI, Henderson CJ, Song L, Thornalley PJ, et al. Transcription factor Nrf2 is essential for induction of NAD(P)H:Quinone oxidoreductase 1, glutathione S-transferases, and glutamate cysteine ligase by broccoli seeds and isothiocyanates J Nutr. 2004 Dec;134(12 Suppl):3499S-506S.

242. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3410-5.

243. Wagner AE, Ernst I, Iori R, Desel C, Rimbach G. Sulforaphane but not ascorbigen, indole-3-carbinole and ascorbic acid activates the transcription factor Nrf2 and induces phase-2 and antioxidant enzymes in human keratinocytes in culture. Exp Dermatol. 2010 Feb;19(2):137-44.

244. Tanigawa S, Fujii M, Hou DX. Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. Free Radic Biol Med. 2007 Jun 1;42(11):1690-703.

245. Gao SS, Choi BM, Chen XY, Zhu RZ, Kim Y, So H, et al. Kaempferol suppresses cisplatin-induced apoptosis via inductions of heme oxygenase-1 and glutamate-cysteine ligase catalytic subunit in HEI-OC1 cell. Pharm Res. 2010 Feb;27(2):235-45.

246. Nho CW, Jeffery E. The synergistic upregulation of phase II detoxification enzymes by glucosinolate breakdown products in cruciferous vegetables. Toxicol Appl Pharmacol. 2001 Jul 15;174(2):146-52.

247. Allen SW, Mueller L, Williams SN, Quattrochi LC, Raucy J. The use of a high-volume screening procedure to assess the effects of dietary flavonoids on human cyp1a1 expression. Drug Metab Dispos. 2001 Aug;29(8):1074-9.

248. Murray M. Altered CYP expression and function in response to dietary factors: Potential roles in disease pathogenesis. Curr Drug Metab. 2006 Jan;7(1):67-81.

249. Han YL, Yu HL, Li D, Meng XL, Zhou ZY, Yu Q, et al. Inhibitory effects of limonin on six human cytochrome P450 enzymes and P-glycoprotein in vitro. Toxicol In Vitro. 2011 Dec;25(8):1828-33.

250. Nabekura T, Kamiyama S, Kitagawa S. Effects of dietary chemopreventive phytochemicals on P-glycoprotein function. Biochem Biophys Res Commun. 2005 Feb 18;327(3):866-70.

251. Okura T, Ibe M, Umegaki K, Shinozuka K, Yamada S. Effects of dietary ingredients on function and expression of P-glycoprotein in human intestinal epithelial cells. Biol Pharm Bull. 2010 Feb;33(2):255-9.

252. Zhang Y, Callaway EC. High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate. Biochem J. 2002 May 15;364(Pt 1):301-7.

253. Sibhatu MB, Smitherman PK, Townsend AJ, Morrow CS. Expression of MRP1 and GSTP1-1 modulate the acute cellular response to treatment with the chemopreventive isothiocyanate, sulforaphane. Carcinogenesis. 2008 Apr;29(4):807-15.

254. Borska S, Sopel M, Chmielewska M, Zabel M, Dziegiel P. Quercetin as a potential modulator of P-glycoprotein expression and function in cells of human pancreatic carcinoma line resistant to daunorubicin. Molecules. 2010 Feb 9;15(2):857-70.

255. Choi JS, Piao YJ, Kang KW. Effects of quercetin on the bioavailability of doxorubicin in rats: Role of CYP3A4 and P-gp inhibition by quercetin. Arch Pharm Res. 2011 Apr;34(4):607-13.

256. Chieli E, Romiti N, Rodeiro I, Garrido G. In vitro modulation of ABCB1/P-glycoprotein expression by polyphenols from mangifera indica. Chem Biol Interact. 2010 Aug 5;186(3):287-94.

257. van Zanden JJ, van der Woude H, Vaessen J, Usta M, Wortelboer HM, Cnubben NH, et al. The effect of quercetin phase II metabolism on its MRP1 and MRP2 inhibiting potential. Biochem Pharmacol. 2007 Jul 15;74(2):345-51.

258. Gyemant N, Tanaka M, Antus S, Hohmann J, Csuka O, Mandoky L, et al. In vitro search for synergy between flavonoids and epirubicin on multidrug-resistant cancer cells. In Vivo. 2005 Mar-Apr;19(2):367-74.

259. An G, Gallegos J, Morris ME. The bioflavonoid kaempferol is an Abcg2 substrate and inhibits Abcg2-mediated quercetin efflux. Drug Metab Dispos. 2011 Mar;39(3):426-32.

260. Koh E, Wimalasiri KMS, Chassy AW, Mitchell AE. Content of ascorbic acid, quercetin, kaempferol and total phenolics in commercial broccoli. Journal of Food Composition and Analysis. 2009;22(7-8):637-43.

261. Finley JW. Reduction of cancer risk by consumption of selenium-enriched plants: Enrichment of broccoli with selenium increases the anticarcinogenic properties of broccoli. J Med Food. 2003 Spring;6(1):19-26.

262. Iqbal MCM, Röbbelen G, Möllers C. Biosynthesis of glucosinolates by microspore derived embryoids and plantlets in vitro of brassica napus L. Plant Science. 1995 11/24;112(1):107-15.

263. Neave AS, Sarup SM, Seidelin M, Duus F, Vang O. Characterization of the N-methoxyindole-3-carbinol (NI3C)--induced cell cycle arrest in human colon cancer cell lines Toxicol Sci. 2005 Jan;83(1):126-35.

264. Toler HD, Charron CS, Sams CE, Randle WR. Selenium increases sulfur uptake and regulates glucosinolate metabolism in rapid-cycling brassica oleracea. J Am Soc Hort Sci. 2007;132(1):14-9.

265. Kim HS, Juvik JA. Effect of selenium fertilization and methyl jasmonate treatment on glucosinolate accumulation in broccoli florets. J Am Soc Hort Sci. 2011;136(4):239-46.

266. Baker RD,Jr, Baker SS, Rao R. Selenium deficiency in tissue culture: Implications for oxidative metabolism J Pediatr Gastroenterol Nutr. 1998 Oct;27(4):387-92.