Chemoprevention Study of Botanical Dietary Supplements by Mass Spectrometry

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THESIS

Submitted as partial fulfillment of the requirements
For the degree of Doctor of Philosophy in Pharmacognosy
in the Graduate College of the
University of Illinois at Chicago, 2012

Chicago, Illinois

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This thesis is dedicated to my parents, Zhengxing Qiu and Zaiqing Xiang, without whom it would never been accomplished.

ACKNOWLEDGEMENTS

My sincere gratitude goes to my advisor Dr. Richard B. van Breemen, for his support, instruction, encouragement and patience throughout my Ph. D research. I really appreciate his time and assistance in helping me with my research, manuscript, presentation at academic meetings and this dissertation. His guidance and understanding in the last five years will be deeply remembered through my life.

I thank my dissertation committee members, Dr. Brian Murphy, Dr. Scott Franzblau, Dr. Jimmy Orjala and Dr. Maarten Bosland for their invaluable comments and suggestions in my research.

I thank Ms. Yang Yuan for her help, discussion and collaboration in all the experiments during these five years. I would also like to thank Dr. Dejan Nikolic for his insightful discussion and critical suggestions throughout my research, for sharing his broad knowledge in drug metabolism and mass spectrometry.

I thank Dr. Guido Pauli and Dr. Shaonong Chen for providing the hops clinical extract and pure prenylflavonoids for my graduate research.

I thank Dr. Larisa Nonn for providing precious prostate primary epithelial cells and Avani for culturing these cells.

Without the help of my fellow graduate students and many other people my work would never be accomplished. Many thanks go to Dr. Yongsoo Choi, Dr. Hongmei Cao, Dr. Jeff Dahl, Dr. Jerry White, Dr. Shunyan Mo, and Mr. Linlin Dong, Mr. Rui Yu, Dr. Chenqi Hu, Dr. Dongting Liu, Dr. Carrie Crot for their generous help and suggestions in these five years and all the other

ACKNOWLEDGEMENTS (Continued)

members in Dr. van Breemen's lab and staff in the Department of Medicinal Chemistry and Pharmacognosy for their assistance during my time in here.

Thanks NIH/UIC Botanical Center for providing support for my graduate research work.

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LIST OF ABBREVIATIONS

8-PN	8-prenlynaringenin
6-PN	6-prenlynaringenin
AHR	Aryl hydrocarbon receptor
AKT	Protein kinase B
AR	Androgen receptor
ARE	Antioxidant response element
BP	Binding protein
BPH	Benign prostatic hyperplasia
CID	Collision induced dissociation
CLIC1	Chloride intracellular channel protein 1
Cx43	Connexin 43
CYP	Cytochrome P450
DDI	Drug–drug interaction
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GO	Gene ontology
GP	Glutatione peroxidase

LIST OF ABBREVIATIONS (Continued)

GR	Glutathione reductase
Grp 78	78 kDa Glucose-regulated protein
GSH	Glutathione
GST	Glutathione-S-transferase
GSTO1	Glutathione-S-transferase omega 1
GSTP1	Glutathione-S-transferase P1
HAA	Heterocyclic aromatic amine
HBSS	Hanks balanced salt solution
HLM	Human liver microsomes
HSP	Heat shock protein
HSP 27	Heat shock protein beta 1
HSP 90	Heat shock protein 90
IGF	Insulin-like growth factor
IL-6	Interleukin-6
iTRAQ	Isobaric tag for relative and absolute quantitation
IX	Isoxanthohumol
KCl	Potassium chloride
KCIP1	Protein kinase C inhibitor 1
LTQ	Linear ion trap
MAPK	Mitogen activated protein kinase
MEKK	MAPK kinase

LIST OF ABBREVIATIONS (Continued)

$MgCl_2$	Magnesium chloride
MIF	Macrophage migration inhibitory factor
MS-MS	Tandem mass spectrometry
mTOR	Mammalian target of rapamycin
NCE	New chemical entity
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate buffered saline
PC	Prostate cancer
PI3K	Phosphatidylinositol 3-kinases
PKM2	Pyruvate kinase isozyme M2
PQD	Pulsed Q dissociation
PrEGM	Prostate epithelial cell growth medium
PSA	Prostate specific antigen
PXR	Pregnane X receptor
QR	Quinone reductase
REDUCE	Reduction by dutasteride of prostate cancer events
RPS3	40S Ribosomal protein S3
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate

LIST OF ABBREVIATIONS (Continued)

SFN	Epithelial cell marker protein 1
TCTP	Translationally-controlled tumor protein
TNF	Tumor neurosis factor
TXNDC17	Thioredoxin domain-containing protein 17
TyrRS	Tyrosyl-tRNA synthetase
XN	Xanthohumol
UHPLC	Ultra high pressure liquid chromatography

SUMMARY

Prostate cancer ranks first in cancer incidence in American men and it is the second leading cause of cancer related death in the United States. Every year there are more than 230,000 men diagnosed with prostate cancer and more than 30,000 men die with it. American men have a life time risk of 18% to be diagnosed with prostate cancer. African Americans have the highest risk to develop prostate cancer followed by Caucasians, it is not very common in Asian populations which makes us believe that diet may play an important role in preventing prostate cancer.

Lycopene is an antioxidant carotenoid giving tomato its red color. Different lycopene chemopreventive mechanisms have been postulated based on epidemiological studies, human clinical trials, animal model studies, human and animal normal cell and cancer cell line culture. These studies suggest that lycopene can prevent prostate cancer at different stages including initiation, promotion and progression. Since various mechanisms of action were found in different experiment systems, the purpose of this dissertation was, in part, to explore the human prostate primary epithelial cells proteome to see if all those chemopreventive effects can be seen in this type of cell culture, which are believed to be the origin of prostatic adenocarcinomas. Using an iTRAQ quantitative proteomics technique reported in Chapter 2, we were able to quantify more than 400 proteins. Among the proteins that were significantly up-regulated or down-regulated were those involved in antioxidant activation, cyto-protective enzymes upregulation, apoptosis induction, growth inhibition, androgen receptor signaling reduction, and Akt/mTOR pathway down regulation.

SUMMARY (Continued)

Humulus lupulus L. (hops) is well-known as a raw material in the brewing industry to give beer the signature aroma and flavor. Traditionally, hop preparations have been used to treat sleeping disorders, nervousness, excitability, and as sedative, etc. In recent years prenylated flavonoids in hops attracted major attention because of their diverse activities. Xanthohumol, the most abundant chalcone from hops, emerged as a potential chemoprevention agent because of its broad activities against different cancer cell lines, especially prostate cancer cells. However, little is known about the safety of whether xanthohumol or hops extract will cause drug-herb interactions in the human body.

Using the methods reported in Chapter 3, we evaluated a hop standardized extract for possible induction of human metabolizing enzymes cytochrome P450 1A2 and 3A4 according to FDA drug-drug interaction guidance for industry. We found that the hop extract will not induce either enzyme or its corresponding mRNA level at average physiological plasma concentrations. In Chapter 4 we further evaluated the inhibition potential of the hop extract or xanthohumol in combination with other major prenylated flavonoids on drug metabolizing enzymes according to the 2006 FDA guidance for industry. Our results indicate that xanthohumol or hop extract could inhibit cytochrome P450 2C family enzymes which may affect the efficacy and safety of some CYP 2C substrate drugs when co-administere. This inhibitory effect on the CYP 2C family should be further studied *in vivo* to evaluate its significance.

CHAPTER 1

INTRODUCTION

1.1 Prostate cancer chemoprevention by lycopene

1.1.1 Prostate Cancer

Prostate cancer is the most frequently diagnosed non-dermatological cancer and the second leading cause of cancer-related death in men in the United States, exceeded only by skin cancer. The life time risk of prostate cancer in American men is one in six. Every year, in the United States approximately 230,000 new cases of prostate cancer are diagnosed and there are 30,000 prostate cancer deaths.

Prostate cancer exhibits huge differences in incidence among populations worldwide. These differences may be due to dietary, genetic, environmental, and social influences which affect the development and progression of the disease (1). Men from southeast Asia typically consume more soy products than American men, and the phytoestrogens in soy might contribute to the low risk of prostate cancer in the Asian population. However, genetics may contribute to prostate cancer risk, knowledge of the molecular genetics of prostate cancer is still limited. More frequent prostate cancer screening in the United States than in Asia might contribute to a higher apparent incidence of prostate cancer in Americans (2).

The three most important risk factors for prostate cancer in the United States are age, race and family history. Prostate cancer is rarely seen in men younger than 40 years old. The probability of prostate cancer diagnosis in men younger than 40 is 1 in 8,499, for men aged from 40 to 59 is 1 in 38, for men aged from 60 to 69 years is 1 in 15, and for men aged 70 years

and older 1 in 8, with an overall lifetime risk of prostate cancer diagnosis is 1 in 6 (3). About 5% to 10% of prostate cancer cases are believed to be caused by inherited genetic factors or some susceptible prostate cancer genes. Several large case-control studies and cohort studies from various populations suggest that family history is a major risk factor in prostate cancer (4-6). Asian men usually have a low incidence with rates ranging from 2 to 10 per 100,000 men. Higher incidence rates are generally observed in northern European countries. African American men have the highest incidence of prostate cancer in the world. In the United States, African American men have a 60% higher incidence than white men (7).

Transformation of normal prostate epithelial cells and progression to prostate carcinoma can result from a series of initiation and promotional stages under genetic, epigenetic and environmental influences (8). Prostate cancer carcinogenesis is a multiple step process induced by genetic and/or epigenetic changes that disrupt molecular pathways involved in cell proliferation, differentiation, apoptosis, and senescence (9). Several precursor lesions in prostate tissue contain genetic changes representing intermediate stages between normal and malignant epithelium (10). These lesions may appear 20 years before the appearance of clinical prostate cancer. Theoretically, the extended period of time required for development of prostate cancer provides an opportunity to intervene before malignancy is established.

1.1.2 Chemoprevention of prostate cancer

Chemoprevention is defined as the prevention of cancer initiation, promotion, or progression by administering dietary or pharmaceutical agents (11, 12). With the slow promotion and progression from precancerous lesions to clinical cancer, chemoprevention is

ideal for prostate cancer intervention. Since prostate carcinogenesis is a multistep process, and a number of molecular targets are associated with its initiation, promotion and progression, a variety of possible chemoprevention strategies can be used (9). The roles of hormonal environment and diet are two major directions of research. Several promising agents for prostate prevention including agents targeting androgen metabolism like 5α-reductase inhibitors, and dietary supplements like lycopene, vitamin E and selenium.

The testosterone derivative dihydrotestosterone (DHT) has been recognized as the major regulator of growth in normal and neoplastic prostate tissue. 5-α-Reductase is the enzyme responsible for conversion of circulating testosterone to DHT which causes prostate epithelial cell proliferation. Inhibition of 5-α-reductase decreases the amount of DHT in prostate tissue, and thereby lowers androgenic stimulation of cell proliferation in prostate tissue. *In vitro* studies suggest that 5-α-reductase inhibition slows the growth of previously established prostate cancer cells (13). In studies of prostate cancer tumors grafted into animals, 5-α-reductase inhibition impedes tumor implantation and growth (14).

Finasteride was the first 5-α-reductase inhibitor to enter human trials showed a few side effects, and was found to be effective in the treatment of moderate benign prostatic hyperplasia (BPH). Dutasteride is an alternative 5-α-reductase inhibitor that was evaluated in the REDUCE (Reduction by Dutasteride of Prostate Cancer Events) trial. DHT synthesis from testosterone is catalyzed by 5-α-reductase types I and II. Finasteride selectively inhibits the type II isoenzyme, whereas dutasteride is a dual inhibitor. Dutasteride suppresses DHT 90% to 94% compared with 67% to 76% for finasteride (15). However, both drugs were disapproved by the FDA in December 2010 because they were linked to a increased incidence of high-grade tumors.

Selenium is a micronutrient found in many vegetables and grains; and it has anti-proliferative and antioxidant properties that may protect against prostate cancer (16). Selenium has also been shown to induce apoptosis, inhibit cellular proliferation and inhibit angiogenesis. Reductions in the incidence of prostate cancer have been reported by up to 50–65% for high versus low selenium levels (17). However, no reduction in risk was found with long-term intake of > 50 ng of selenium per day (18). A meta-analysis of 20 epidemiological studies showed a significant increase in the incidence of prostate cancer in men with low selenium levels (19). The findings of these studies are supported by a randomized interventional trial of cancer prevention, where the primary end point was nonmelanoma skin cancer, showing an approximately 50% reduced risk of prostate cancer with a daily 200 μg selenium supplement, particularly in men with low selenium levels (20-22). However, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) found no reduction in the risk of prostate cancer with either selenium or vitamin E supplementation. A follow-up study even found that dietary supplementation with vitamin E can significantly increased the risk of prostate cancer among healthy men (23).

Lycopene is the most abundant carotenoid in tomatoes and tomato-derived products, and is the most potent antioxidant among the more than 600 naturally occurring carotenoids. Lycopene consumption was inversely associated with the occurrence of prostate cancer in case–control studies (24, 25) and tomato intake and serum lycopene levels were linked with a reduced incidence of advanced prostate cancer, or the prevention of progression of prostate cancer in three case–control studies (26-28). Beneficial associations were observed for tomato or vegetable juice and ketchup in one study (26). A meta-analysis of ten cohort and eleven

case–control studies found an association between lycopene intake and decreased risk of prostate cancer (29). In the Health Professionals Follow-Up Study, 2–4 weekly servings of tomato sauce reduced risk of total prostate cancer by one-third and aggressive prostate cancer by almost one-half, and there were no reported adverse effects (30).

1.1.3 Chemoprevention by lycopene

Lycopene is a non–provitamin carotenoid that gives tomatoes their red color (Figure 1). It is a potent antioxidant and quencher of singlet oxygen (31), resulting in protection against

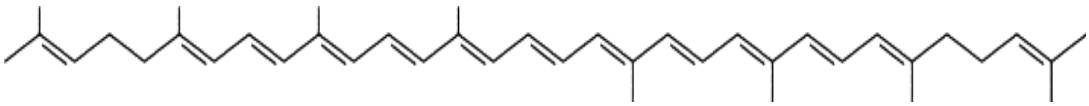


Figure 1. All trans Lycopene

oxidative DNA damage *in vitro* and *in vivo* (32). Evolving evidence suggests that lycopene may modulate processes related to mutagenesis, carcinogenesis, cell differentiation, and proliferation independently of their role as antioxidants (33). Epidemiological studies have shown an inverse association between dietary intake of tomatoes, tomato products, lycopene, and prostate cancer risk (34). The results from a recent meta-analysis suggest a 25% to 30% reduction in the risk of prostate cancer with tomato, tomato products, and lycopene intake (29). Rao *et al*. observed significantly lower serum and prostate tissue lycopene levels in patients with prostate cancer than in controls in a case-control study (35). Lycopene and β-carotene were the predominant carotenoids in human prostate tissue obtained by radical prostatectomy

from men with prostate cancer (36). Table 1 (reprint with permission) summarizes the type and duration of intervention and the outcome measures in lycopene clinical studies.

Lycopene has been reported to increase the activity of the Phase II enzymes quinone reductase (QR), glutathione peroxidase (GP), glutathione reductase (GR), glutathione (GSH), and glutathione-S-transferase (GST) in several animal models (37-42). Induction of Phase II enzymes was associated with suppressed DMBA-induced oral carcinogenesis in hamsters (40). Lycopene also reduces lipid peroxidation (39-41). Genes encoding Phase II enzymes and the oxidative defense system are co-regulated by the Nrf2/Keap1 system via the antioxidant response element (ARE) in the promoter region of these genes.

Ben-Dor *et al*. showed that lycopene, and possible lycopene metabolites, are capable of activating ARE in an Nrf2-dependent manner (43). Tomato intervention in humans for 60 days significantly increased the serum levels of the Phase II enzymes and GSH (44). Up regulation of Phase II enzymes indicates that lycopene improves the protection against exogenous toxins including carcinogens.All these *in vitro*, *in vivo* and clinical trials support the hypothesis that lycopene, the most abundant carotenoid in tomato, induces the expression of cytoprotective enzymes in human prostate and functions as a prostate cancer chemoprevention agent.

TABLE I

LYCOPENE CLINICAL TRIALS AND OUTCOMES (45)

Study design	Sample size	Stages of cancer	Dose per day	Form	Duration	Outcome	Reference
Before-after, no control group	46	Androgen refractory	30 mg	Tomato paste/juice	16 weeks	Changes in PSA, survival toxicity	Jatoi *et al*.(46)
Before-after, no control group	32	Stage T1 or T2	30 mg	Tomato sauce	3 weeks	Changes in PSA	Chen *et al*.(47)
Before-after, no control group	20	Metastatic with hormone refractory	10 mg	Lycored softules	12 weeks	Changes in PSA, urinary tract symptoms, bone pain	Ansari *et al*.(48)
Before-after, no control group	37	Localized	10 mg	Lycopene tablet	1 year	Changes in PSA, toxicity	Barber *et al*.(49)
Before-after, no control group	36	Biochemically recurrent	15/30/45/ 60/120 mg	Lycopene capsule	1 year	Changes in PSA, toxicity	Clark *et al*.(50)
RCT	27 intervention 27 controls	Metastatic	4 mg	Not reported	2 years	Changes in PSA, bone metastasis survival	Ansari *et al*.(51)
RCT	15 intervention 11 controls	Stage T1 or T2	30 mg	Lycopene capsule	3 weeks	Changes in PSA	Kucuk *et al*.(52)
NRCT	32 intervention 34 controls	Stage T1 or T2	30 mg	Tomato sauce	3 weeks	Changes in PSA	Kim *et al*. (53)
RCT	69 intervention 62 controls	Prostate cancer or BPH	30 mg	Lycored capsule	3 weeks	Reduction in 8-oxo-dG, PSA	van Breemen et al. (10)

RCT, randomized controlled trial; NRCT, non-randomized controlled trial.

1.1.4 Mechanisms of lycopene function

Postulated mechanisms of action of lycopene include inhibition of growth and induction of differentiation in cancer cells by modulating the expression of cell cycle regulatory proteins (54); modulation of the insulin-like growth factor (IGF)-1/IGF binding protein (BP)-3 system (55-57); up regulation of gap-junction gene connexin 43 (Cx43) and increased gap junction intercellular communication (58, 59); balancing redox signaling (60); prevention of oxidative DNA damage (35, 61); inhibition of interleukin (IL-6) and androgen (62); inhibition of 5-lipoxygenase (63); up regulating carcinogen-metabolizing enzymes (64); and modulation of immune function (65) inducing phase II protective enzymes(66) (Figure 2, reprint with permission). These mechanisms might be interrelated and might operate synergistically.

Lycopene inhibits IGF-I signaling at different stages of the signaling pathway. In LNCaP cells, lycopene was found to decrease IGF1R expression and activation, decrease protein kinase B (AKT) activation, and increase IGFBP2 expression (67). In prostate tissue of healthy rats (68) and in rat prostate tumors (62), lycopene was reported to decrease the local expression of IGF-I mRNA. Lycopene reduces androgen signaling in reactive prostate stromal cells by decreasing DHT-induced IGF-I production in the cells and reducing DHT-induced nuclear transportation of androgen receptor (AR) and of *β*-catenin (69). In healthy rat prostate tissue, 5-*α*-reductase II and androgen target genes, such as seminal vesicle secretion protein IV, cystatin related protein 2, and prostatic steroid binding protein C1 and C3 were down-regulated (68). In rat prostate tumors, lycopene reduced expression of 5-*α*-reductase I and androgen target genes such as prostatic spermine-binding protein, cystatin related protein 1 and 2, probasin, and prostatic steroid-bg protein C1, C2, and C3 chain (62).

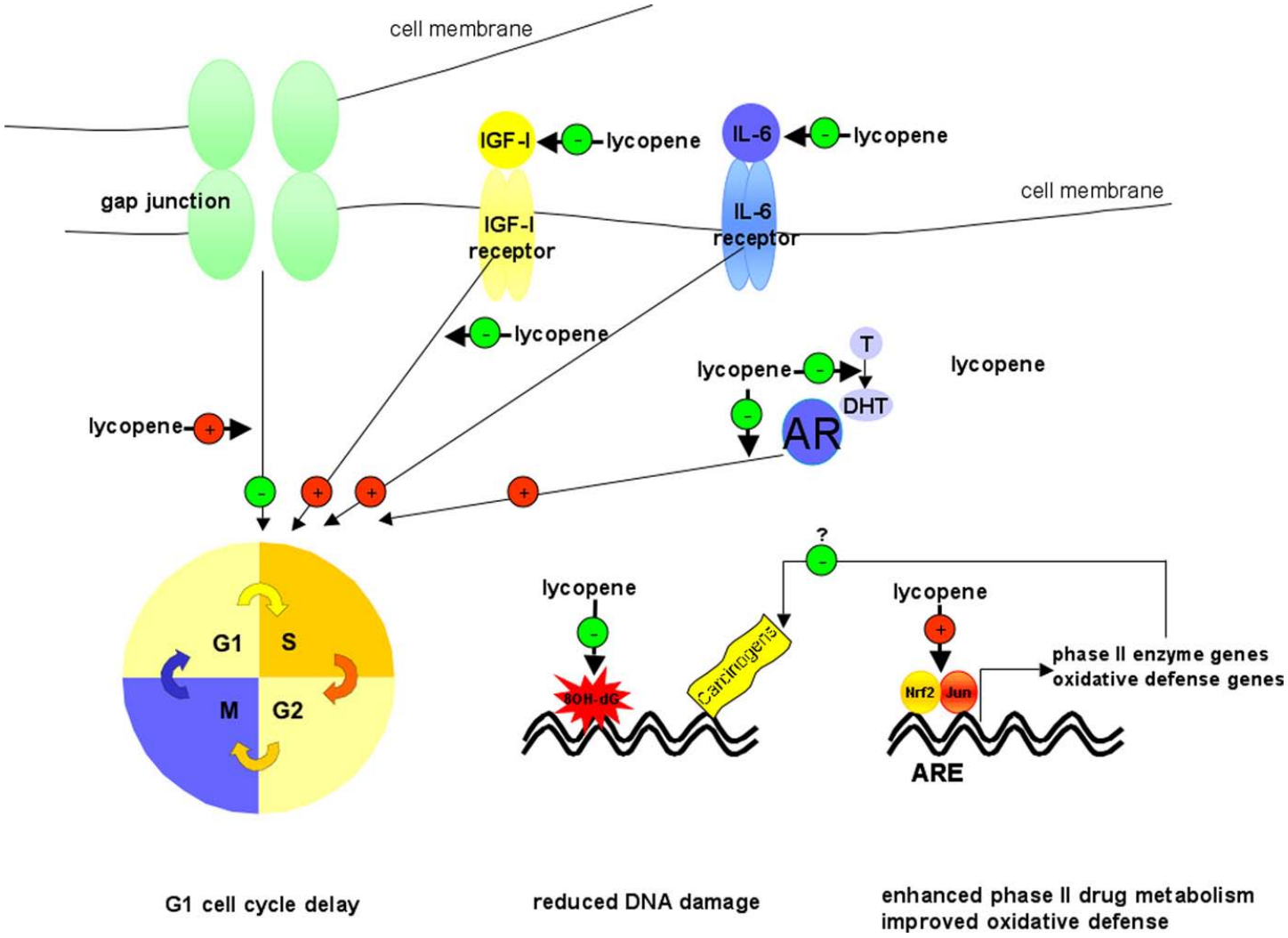


Figure 2. Lycopene might reduce prostate cancer risk through multiple mechanisms (70).

Lycopene has also been reported to down-regulate inflammatory regulators, such as cytokines, enzymes, and transcription factors. Lycopene decreased expression of IL-1*β*, MIP-2, and LIX in healthy rat prostate tissue, which are markers for immune cell infiltration (68). In rat prostate tumors , lycopene showed anti-inflammatory activity by decreasing IL-6 expression (62) and by preventing NF-*κ*B activation to (71). These anti-inflammation effects of lycopene should reduce the probability of prostate cancer progression to androgen-independent growth.

The cell membrane contains lipid rafts which are rich in cholesterol and sphingolipids, and contain receptors and other proteins required for signal transduction. Relevant to prostate cancer, nongenomic androgen receptor signaling depends on lipid rafts (72). In membrane models, lycopene incorporation into cholesterol containing model membranes led to membrane disorganization (73). Thus, lycopene could inhibit signaling pathways also by interfering with lipid raft-associated signaling processes. All these findings strongly support the hypothesis that lycopene may prevent prostate cancer.

1.2 iTRAQ quantitative proteomics

Identification and quantitation of biologically important proteins is usually carried out using immunoassays, such as western blot and ELISA. With the advances of high-resolution mass spectrometry and bioinformatics, qualitative and quantitative analysis of thousands of proteins may now be carried out during a simple analysis. Although these techniques still do not allow definitive analysis of the entire proteome of a cell, the relative quantitative analysis of proteins in complex mixtures from cells provides a means to understand molecular changes that occur during perturbation of the cell system.

iTRAQ stands for isobaric tags for relative and absolute quantification and was a chemical reagent that reacts with primary amine groups on Lysine (Lys) residues and N-terminal of peptides. The structure of the iTRAQ reagent, shown in Figure 3, has a peptide reactive group and an isobaric tag. Upon reaction with peptide, the isobaric tag, total mass of 145, will be incorporated. The tag consists of a reporter group with a mass of 114 to 117, and a balance group with a mass of 28 to 31. So an iTRAQ 114 reagent means it has reporter group of mass 114 and a balance group of 31, while iTRAQ 117 reagent means it has a reporter group of mass 117 and balance group of 28. The iTRAQ reagent derivatizes peptides quantitatively without major side reactions. Peptide labeled with iTRAQ reagents with different masses coelute during liquid chromatography mass spectrometry (LC-MS-MS) but can be distinguished by mass.

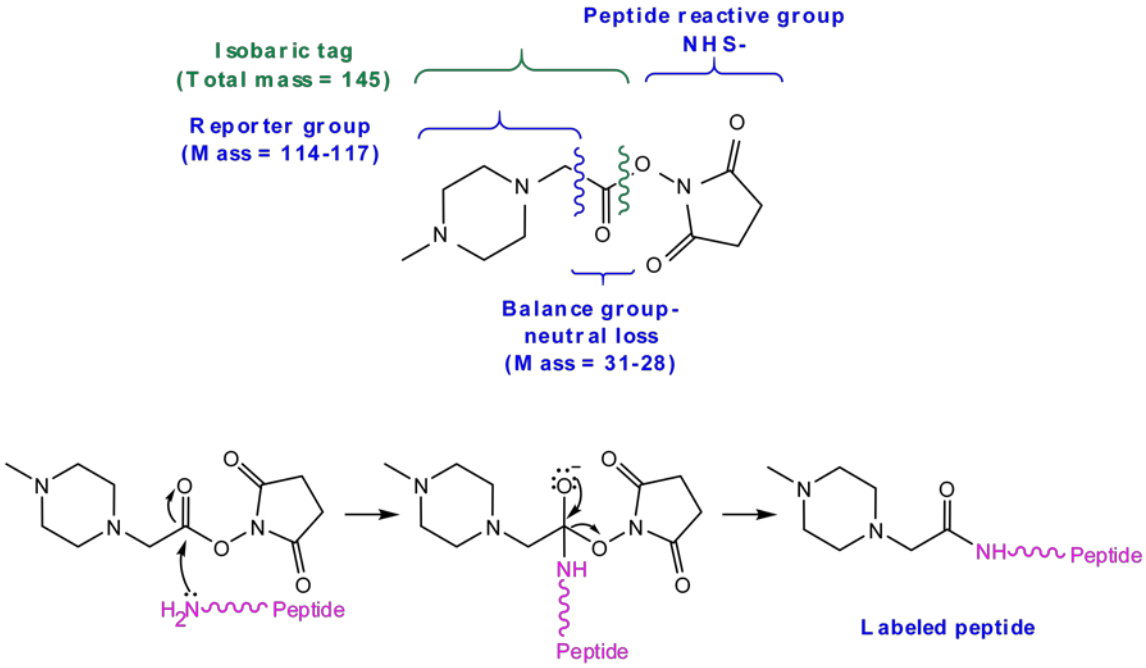


Figure 3. iTRAQ reagent structure and reaction with peptides.

The iTRAQ quantitative proteomics work flow is shown in Figure 4 (74). First, cell cultures are treated with a test agent or special incubation condition or a control experiment. After treatment is completed, the cells are harvested, the proteins are extracted and the concentration of protein from each treatment group is determined.

Any disulfide bonds are reduced by tris-(2-carboxyethyl)phosphine (TCEP) and the reduced cysteines are then deactivated by methyl methanethiosulfonate (MMTS) to prevent disulfide bond reformation. The reduced proteins are then digested by trypsin, which cleaves on carboxyl side of lysine and arginine except when followed by proline. The protein to enzyme ratio should be between 100:1 to 10:1, and digestion should be carried at 37℃ overnight with shaking. The tryptic peptides from different experiments are labeled with iTRAQ reagents of different masses. After labeling, the proteins from different experiment will be combined together and analyzed by LC-MS/MS with or without further sample clean up depending on the sample complexity.

During LC-MS/MS analysis, peptides are detected and sequenced. A database search engine assigns each peptide to a specific protein for protein identification. Peptides from each sample are differentially labeled with iTRAQ reagent but elute together during LC-MS/MS as illustrated in Figure 5 (reprint with permission) (75). During MS/MS , the co-eluting peptide produce the same *b* ions and *y* ions which give the peptide amino acid sequence, but in the lower mass region from 114 to 117, the relative abundances of the iTRAQ reporter ions indicate their relative amounts.

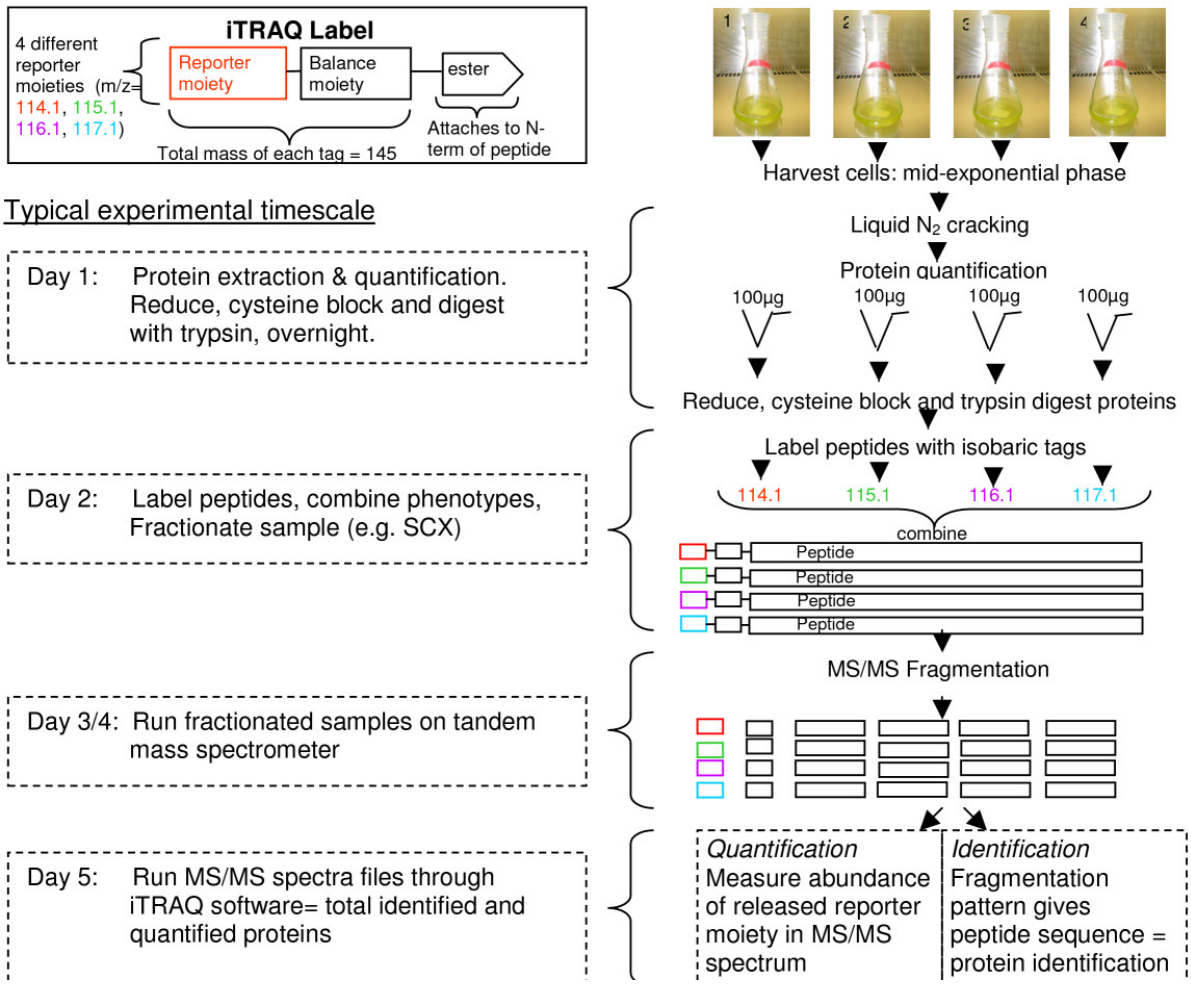


Figure 4. Work flow of iTRAQ quantitative proteomics (74).

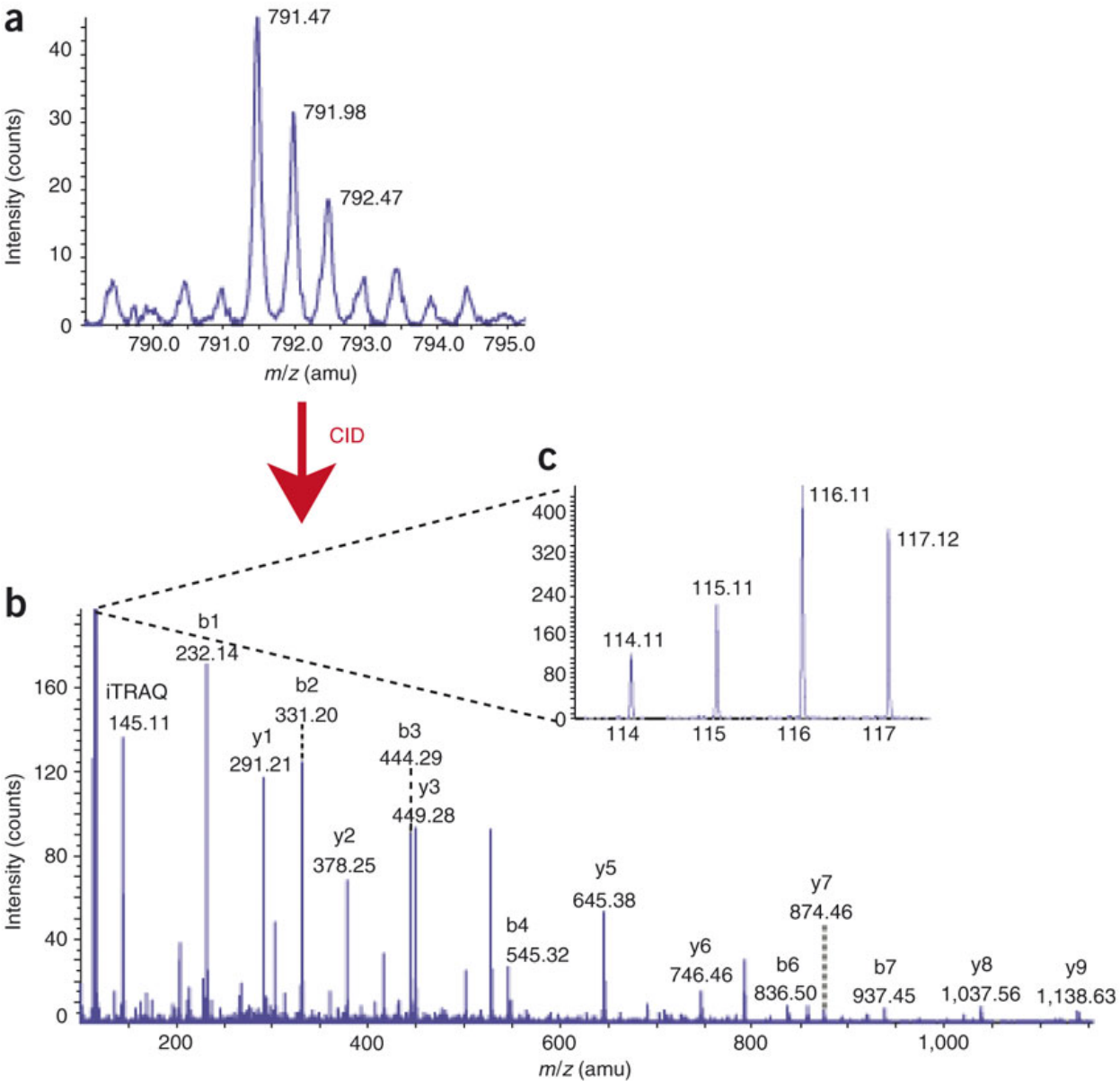


Figure 5. Representative MS and MS/MS spectra of an iTRAQ-labeled peptide (75).

1.3 Xanthohumol from hops as a chemoprevention agent

The female flowers from hops (*Humulus lupulus* L.) are rich in polyphenolic compounds and acyl phloroglucides and are widely used to preserve beer and to give beer its characteristic aroma and flavor. In addition, hop cones have long been used for medical purposes particularly for the treatment of sleeping disorders, as a mild sedative, and to activate gastric function (76). There are more than 1000 chemical constituents in hops, but the volatile oil and bitter acids are the most important compound classes in hops in terms of beer production. The prenylated flavonoids in hops have attracted attention because of their chemoprevention and estrogenic activities (77). The structures of the most abundant and estrogenic prenylated flavonoids are shown in Figure 6, xanthohumol (XN), isoxanthohumol (IX), 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN).

1.3.1 Chemoprevention activity of Xanthuhomul

Over the past decade, multiple *in vitro* studies have been reported concerning the activities of hops and hop constituents as chemopreventive agents. Among hop components, XN has shown the most chemoprevention activity including the prevention of initiation, promotion and progression of carcinogenesis in *in vitro* models, and appeared as a broad spectrum chemoprevention agent (78, 79) in *in vivo.* A brief summary of *in vitro* biological activity of hops prenylated flavonoids is shown in Table II.

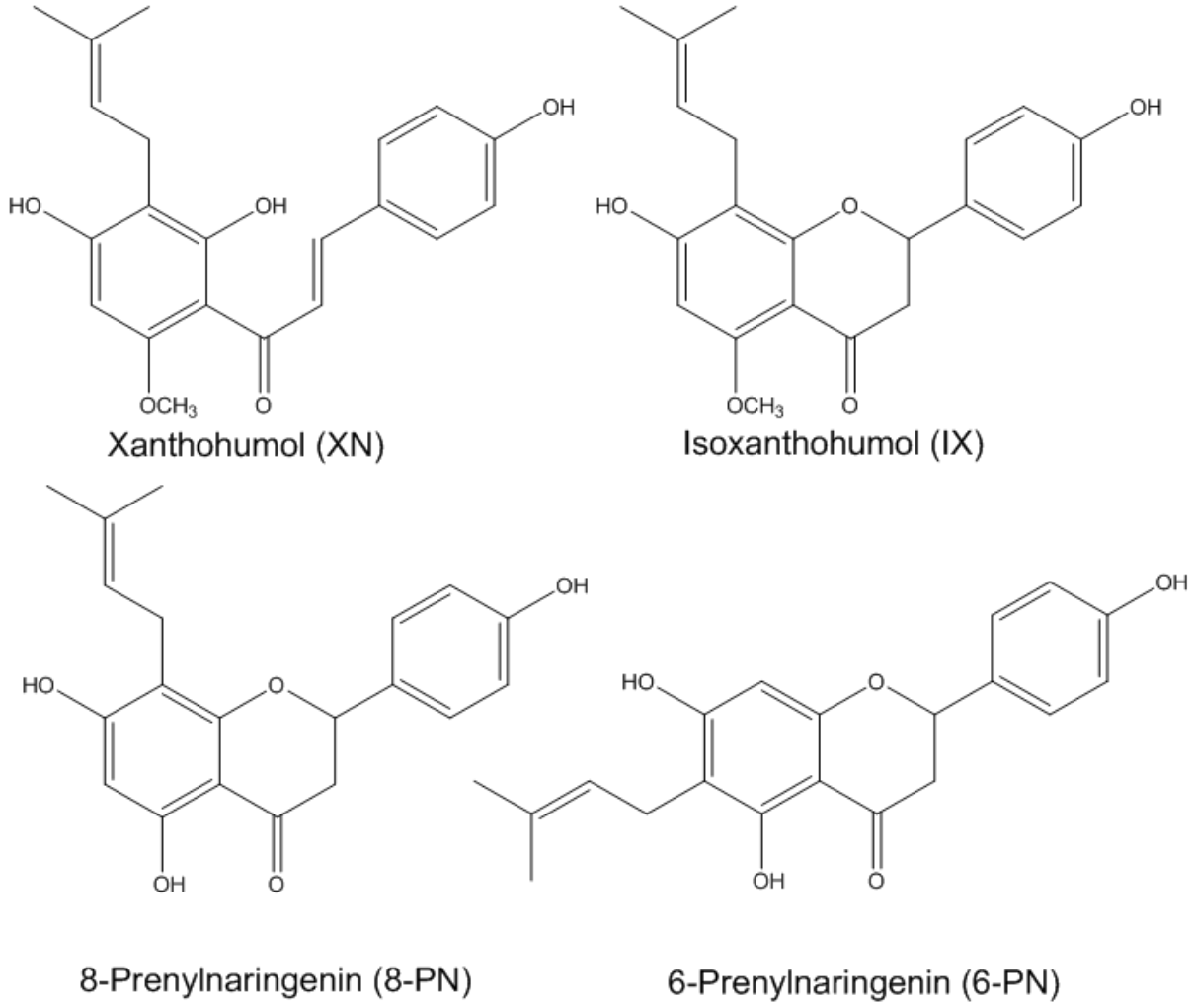


Figure 6. Structures of four important prenylated flavonoids from hops XN, IX, 8-PN, and 6-PN.

TABLE II

IN VITRO CHEMOPREVENTIVE ACTIVITY OF HOPS PRENYLATED FLAVONOIDS (76)

Substrate/cell line	Biological activity	Main active components	IC_{50} values (μM)	Reference
MCF-7, HT-29, A2780	Antiproliferative activity	XN, IX	0.5-15	Miranda *et al*. (80)
BPH-1, PC-3	Induction of apoptosis	XN	10-20	Colgate *et al*. (81)
Human placental vessels	Inhibition of angiogenesis	XN, IX	<10	Bertl *et al*. (82)
Isolated human LDL	Antioxidant and antiperoxidant	XN, DMX	5-25	Miranda *et al*. (83)
HUVEC	Antiproliferative activity	XN	<10	Albini *et al*. (84)
Mouse hepatoma heap 1c1c7 cells	Induction of QR activity	XN, 8-PN, 6-PN	1-10	Miranda *et al*. (85)
MCF-7, T47-D	Induction of apoptosis	XN	<10	Vanhoecke *et al*. (86)
PC-3, DU145	Antiproliferative activity	XN, DMX, IX, 8-PN, 6-PN	12-53	Delmulle *et al*. (87)
CYP1A1, CYP1B1, CYP1A2, CYP3A4, CYP2E1	Inhibition of CYP450	XN, IX, 8-PN	0.05-10	Henderson *et al*. (88)
CYP1A2	Inhibition of CYP450	XN, IX, 8-PN	2-10	Miranda *et al*. (89)
CYP1A	Inhibition of CYP450	XN, IX, 8-PN, 6-PN	0.02-0.2	Gerhauser *et al*. (90)
Sheep seminal vesicle	Inhibition of COX1and COX2	XN, 8-PN, IX	16-41.5	Gerhauser *et al*. (90)
Mouse macrophage cells	Inhibition of nitro oxide xynthase (iNos)	XN, IX	12-22	Gerhauser *et al*. (90)
Bovine endothelial cells	Inhibition of iNos	8-PN	3-10	Pepper *et al*. (91)
Human colon cancer cells	Induction of apoptosis	XN	5-15	Pan *et al*. (92)

Recently, Deeb *et al*. demonstrated that prostate cancer cells are highly sensitive to XN at a concentration range of 20-40 μM. XN induced apoptosis in these cancer cells by the inhibition of prosurvival AKT, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mammalian target of rapamycin (mTOR) signaling proteins and NF-kB regulated anti-apoptotic Bcl-2 and surviving. These mechanisms of chemoprevention provided a rationale for clinical evaluation of XN for the treatment of hormone refractory metastatic prostate cancer (93).

Strathmann *et al*. identified mitochondria as a novel cellular target of XN (94). In three human cancer cell lines, BPH-1 and murine macrophages, XN stimulated superoxide anion radical ($O_2^{-}\cdot$) production and disrupted cellular redox balance and mitochondrial integrity leading to apoptosis. These experiments indicated that XN at concentrations from 1.6 μM to 25 μM induced an immediate and transient $O_2^{-}\cdot$ production in mitochondria. Strathmann *et al.* demonstrated that XN treatment causes a rapid breakdown of the mitochondrial membrane potential and cytochrome c release which induces apoptosis. They also confirmed that this apoptosis induction was triggered by XN-induced $O_2^{-}\cdot$ formation. Szliszka *et al*. reported that XN markedly enhances TNFα induced apoptosis and cytotoxicity in prostate cancer cells and confirmed that XN could served as a chemoprevention agent for prostate cancer (95).

Delmulle *et al*. evaluated the anti-proliferative property of XN in human prostate cancer cell lines PC-3 and DU145. They showed that XN inhibited the growth of DU145 cells with IC_{50} values of 12.3 μM for and inhibited PC-3 cells with an IC_{50} value of 13.2 μM (87). Colgate *et al*. reported XN and XN oxidation product (XOP) induce apoptosis and down regulate NF-κB activation in BPH-1 cells and suggested that XN can prevent prostate hyperplasia and prostate

carcinogenesis (81). They demonstrated in both BPH-1 and PC3 cells XN and XOP decreased cell viability in a dose dependent manner from 2.5 μM to 20 μM and increased the formation of early and late apoptotic cells. XN and XOP also induced cell cycle changes in both cells lines with elevated sub G1 peak at 48 h treatment, active ated proapoptotic proteins Bax and p53 and decreased the activation of NF-κB.

All the above evidence strongly supports that XN, a prenylated flavonoid from hops, is able to inhibit prostate cancer cell growth, enhance apoptosis in prostate cancer cell lines through different mechanisms and also inhibit NF-κB activation to function as a prostate cancer chemoprevention agent.

XN has also been shown to have preventive activities against several other type of cancer cell lines. Zajc *et al*. reported that XN could induce a higher rate of apoptosis in glioblastoma cells than in normal astrocytes with p53 activation and an elevated Bax/Bcl-2 ratio in glioblastoma cells but with reduced Bax/Bcl-2 ratio in normal human astrocytes. This was also linked to higher expression of the cell cycle inhibitor, p21, in glioblastoma cells than in normal astrocytes. They concluded that XN can induce different cytotoxicity and apoptotic pathways in malignant and normal astrocytes and should be investigated as adjuvant therapy agent to treat glioma (96).

Benelli *et al*. found XN to be a potent anti-lymphotytic leukemia agent which can overcome chemoresistance (97). They treated acute lymphocytic leukemia (ALL) cell lines with XN which resulted in growth arrest and apoptosis induction. Moreover, administration of 50 μg XN/mouse (5 days/week) significantly increased animal life span by delaying the occurrence of neurological disorders due to leukemic cell dissemination. XN also significantly down regulated

AKT and NF-kB signaling pathways. Festa *et al*. reported that XN induced apoptosis in human malignant glioblastoma cells by increasing ROS and activating MAPK pathway, suggesting that XN might be a potential chemotherapeutic agent for the treatment of glioblastoma multiforme (98). Hemachandra *et al*. showed that hops extracts possess cancer chemopreventive activity through attenuation of estrogen metabolism by inhibiting estrogen-induced expression of CYP1B1 and 1A1 in MCF-10A cells (99).

Wang *et al*. demonstrated that XN suppressed Cysteine X Cysteine chemokine receptor 4 (CXCR4) expression in various types of cancer cells in a concentration and time-dependent manner. Both proteasome and lysosomal inhibitors had no effect on the prevention of XN-induced downregulation of CXCR4, suggesting that the inhibitory effect of XN was not due to proteolytic degradation but occurred at the transcriptional level. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay further confirmed that XN could block endogenous activation of nuclear factor kappa B, a key transcription factor that regulates the expression of CXCR4 in cancer cells (100).

Viegas *et al*. reported that XN was not mutagenic in *Salmonella typhimurium* TA98 and did not induce genomic instability in human hepatoma HepG2 cells. XN suppressed the formation of 2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine and 2-amino-3,8 dimethylimidazo-[4,5-f] -Quinoxaline induced mutations in a dose dependent manner in bacteria, and in HepG2 cells, it completely prevented DNA strand breaks at nanomolar concentrations of these compounds. Real-time PCR gene expression analysis found that XN could up regulate the expression of phase I (CYP1A1 and CYP1A2) and phase II (UGT1A1) enzymes which may be an important mechanism of XN chemoprevention against carcinogenesis. These findings confirmed that XN

can protect against heterocyclic aromatic amine induced genotoxicity and provide additional mechanistic information to assess its potential chemopreventive effects (101).

Albini *et al*. showed that when XN was administered in the drinking water at 2 μM it strongly inhibited angiogenesis in mice implanted with a matrigel sponge. At 200 μM, XN displayed significant angiogenesis inhibition without adverse effects. If XN was administrated orally at 20 μM, starting from the 20th day of treatment it significantly inhibited the growth rate of KS-IMM tumors in male nude mice (84). XN at 1000 mg/kg/day SC for 14 days inhibit tumor angiogenesis and growth in female immuno-deficient mice implanted with human breast cancer tumor xenografts (79).

XN shows broad chemopreventive activity against different cancer cell lines through different mechanisms including inhibiting metabolic activation of procarcinogens, inducing carcinogen detoxification enzymes, inducing apoptosis, inhibiting tumor cell growth, and interfering with signaling pathways. Further *in vivo* studies to evaluate its chempreventive function is needed.

1.3.2 Estrogenic activity of hops

The menstrual disturbances observed in female hop-pickers, during the early days of hop cones harvesting, suggested potential hormonal activity of hops. In Germany, hop baths have been traditionally used to treat gynaecological disorders (76). Milligan *et al*. reported that among the hop polyphenolic compounds, 8-PN displayed most estrogenicity base on the stimulation of alkaline phosphatase activity in Ishikawa cells, and binding to estrogen receptors in a radio ligand binding assay using rat uterine cytosol (102). These findings were later

confirmed by the same group using a yeast screen expressing the human estrogen receptor (103). The high estrogenic activity of 8-PN was also confirmed in different *in vivo* experiments by other researchers. The subcutaneous administration of 8-PN (30 mg/kg/day) for 2 weeks was reported to slow the decrease in bone mineral density and the reduction in uterine weight ovariectomized rats (104). 8-PN was also reported to induce a characteristic estrogenic response in ovariectomized female rats using uterine vascular permeability as an endpoint (105) and in a 3-day uterotrophic assay (106). At the UIC/NIH Center for Botanical Dietary Supplements, Liu *et al*. reported that a methanol extract of hops showed strong binding affinity to both estrogen receptors (ERα and ERβ), induced alkaline phosphates activity in Ishikawa cells, up-regulated progesterone receptor mRNA in Ishikawa cells, and up-regulated presenelin-2, an estrogen-inducible gene in S30 cells (107).

The first randomized, double blind and placebo-controlled study of a standardized hop extract (standardized to 8-PN) in menopausal women has been published (108). The daily administration of the extract, at a dose equal to 100 µg 8-PN for 6 weeks, to postmenopausal women decreased the frequency of hot flushes frequency and other symptoms due to estrogen deficiency (sweating, insomnia, heart palpitation, irritability). Vaginal dryness in postmenopausal women was significantly reduced by the topical application of a gel containing hyaluronic acid, liposomes, vitamin E and hop extract (109). Rad *et al*. carried out a randomized, double-blind, placebo-controlled study in which a single dose of 8-PN from 50 to 750 mg was administered orally to healthy menopausal women. They found decreased luteinizing hormone (LH) in serum which showed the ability of 8-PN to exert endocrine effects in menopausal women (78). Although large scale clinical studies are needed, the data strongly

suggest that hop-derived prenylated flavonoids might provide an alternative to estrogen replacement therapy for the relief of menopausal symptoms in women.

1.4 Investigation of drug-herb interaction

Drug-herb interaction is a situation in which one affects the activity of the other when taking both together. This interaction can be synergistic or antagonistic or a new effect may be produced. These pharmacological interactions are very important in the practice of medicine because overdose and side effects might occur, and therapeutic effects might disappear. Drug-herb interactions can have pharmacodynamic effects on pharmacological receptors, signal transduction and physiological systems. Drug-herb might also produce pharmacokinetic interaction which can change absorption, distribution, metabolism, and excretion of co-administrated substances. According to the 2006 FDA Drug-Drug Interaction (DDI) industry guidance, DDI studies should carried out to evaluate the inhibition and induction potential of the herb on drug metabolizing enzymes.

Many metabolic routes of elimination, including those through the cytochrome P450 family enzymes system, can be inhibited or induced by concomitant drug treatment. Observed changes arising from metabolic DDI can be substantial, an order of magnitude or more decrease or increase in the blood and tissue concentrations of a drug or metabolite, and can also increase formation of toxic and reactive metabolites or increased exposure of human body to toxic parent compounds. These large changes in exposure could change the safety and efficacy of a drug and/or its active metabolites. This is most obvious and expected for a drug with a narrow therapeutic range (NTR), but is also possible for non-NTR drugs such as HMG

CoA reductase inhibitors (110).

It is important that metabolic DDI studies explore whether an investigational agent is likely to significantly affect the metabolic elimination of drugs which are already in the market and likely in medical practice to be taken concomitantly. On the other hand, whether drugs in the market are likely to affect the metabolic elimination of the investigational drug also need to be evaluated . Even drugs that are not substantially metabolized can have important effects on the metabolism of concomitant drugs. For this reason, metabolic DDI should be explored, even for an investigational compound that is not eliminated significantly by metabolism (110).

A specific objective of metabolic DDI studies is to determine whether the interaction is sufficiently large enough to necessitate a dosage adjustment of the drug itself or the drugs with which it might be used, or whether the interaction would require additional therapeutic monitoring (110).

1.4.1 In intro evaluation of cytochrome P450 (CYP) inhibition

Botanicals that inhibit a specific drug metabolizing enzyme can decrease the metabolic clearance of a co-administered drug that is a substrate of that enzyme. This induction would increase blood concentration of the drug, potentially causing adverse effects or enhanced therapeutic effects. For example, bioactive compounds presented in grapefruit juice and some other fruit juices, including bergamottin, dihydroxybergamottin, and paradicin-A, have been found to inhibit CYP3A4 mediated metabolism of certain medications, such as statins, dihydropyridines, antiarrhythmics, etc., which led to increased bioavailability of these drugs in human body and strong possibility of overdosing and severe side effects (111). On the other

hand, inhibition of metabolizing enzymes could decrease formation of an active metabolite of the co-administered drug which is a prodrug, resulting in decreased efficacy.

For CYP inhibition study, *in vitro* experiments should be conducted to determine whether botanicals inhibit specific CYP enzymes. These experiments usually involve incubation of the drug with probe substrates for specific CYP enzymes. Each probe substrate should be selective for a single CYP in pooled human liver microsomes (HLM) and should have a specific metabolic product without sequential metabolism. The preferred and acceptable substrates based on FDA recommendation are list in Table III (110).

There are some issues concerning the experimental design that should be considered when testing for botanical inhibition of specific CYP enzymes. To determine IC50 value, the substrate concentration in the incubation should be below its Km, and the HLM protein concentration should be below 1 mg/mL. No more than 10-30% substrate depletion should occur during the experiment, there should be a linear relationship between time and metabolite formation and a linear relationship between CYP enzymes amount and metabolite formation (110).

TABLE III

FDA PREFERRED AND ACCEPTABLE SUBSTRATE FOR INHIBITION STUDY (110)

CYP	Preferred Substrate	Km(μM)	Acceptable Substrate	Km (μM)
1A2	Phenacetin	1.7-152	7-Ethoxyresorufin Theophyllin Caffeine Tacrine	0.18-0.21 280-1230 220-1565 2.8, 16
2A6	Coumarin Nicotine	0.30-2.3 13-162		
2B6	Efavirenz Bupropion	17-23 67-168	Propofol S-mephenytoin	3.7-94 1910
2C8	Taxol	5.4-19	Amodiaquine Rosiglitazone	2.4, 4.3-7.7
2C9	Tolbutamide S-warfarin Diclofenac	67-838 1.5-4.5 3.4-52	Flurbiprofen Phenytoin	6-42 11.5-117
2C19	S-mephenytoin 4'-hydroxylation	13-35	Omeprazole Fluoxetine	17-26 3.7-104
2D6	(±)-Bufuralol Dextromethorphan	9-15 0.44-8.5	Debrisoquine	5.6
2E1	Chlorzoxazone	39-157	p-Nitrophenol Lauric acid Aniline	3.3 130 6.3-24
3A4/5	Midazolam Testosterone	1-14 52-94	Erythromycin Dextromethorphan Triazolam Terfenadine Nifedipine	33 – 88 133-710 234 15 5.1- 47

1.4.2 *In vitro* evaluation of cytochrome P450 induction

Botanicals that induce drug metabolizing enzymes can increase the rate of metabolic clearance of a co-administered drug that is the substrate of the induced enzyme. The consequence of this interaction would be decreased therapeutic blood concentrations and lower therapeutic effect. For example, St. John's wort, an antidepressant herb, is a strong inducer of CYP3A4 which can cause low plasma level of certain medication, such as statins, dihydropyridines, antiarrhythmics, etc., resulting in half or even lower co-administered drug efficacy. On the other hand, the induced enzyme could lead to increased formation of a toxic metabolite, possibly resulting in strong adverse effect.

For botanical enzyme induction studies, the experiment should include an acceptable enzyme inducer as control according to FDA guidance. Preferred and acceptable inducers which will increase enzyme activity of probe substrate over 2-fold for CYPs are listed in Table IV (110). These assays usually involve the quantitative determination of CYP enzyme activity in primary hepatocyte culture, either fresh isolated hepatocytes or cryopreserved hepatocytes, following treatment with a positive control inducer, a negative control and the tested articles. Hepatocytes from at least three individual donor should be evaluated because of inter individual differences in enzyme induction potentials. The test article concentration should be based on the expected human plasma concentration. At least three concentrations of the test substances spanning the therapeutic range should be studied including at least one concentration that is an order of magnitude greater than the average expected plasma concentration. If this information is not available, concentrations ranging over at least two orders of magnitude should be studied (110).

TABLE IV

FDA PREFERRED AND ACCEPTABLE CHEMICAL INDUCER FOR *IN VITRO* EVALUATION

CYP	Preferred Inducer	Inducer Concentr-ation (μM)	Fold Induction	Acceptable Inducer	Inducer Concentrat-ions (μM)	Fold Induction
1A2	Omeprazole ß-Naphthoflavone 3-Methylcholanthrene	25-100 33-50 1-2	14-24 4-23 6-26	Lansoprazole	10	10
2A6	Dexamethasone	50	9.4	Pyrazole	1000	7.7
2B6	Phenobarbital	500-1000	5-10	Phenytoin	50	5-10
2C8	Rifampin	10	2-4	Phenobarbital	500	2-3
2C9	Rifampin	10	3-7	Phenobarbital	100	2.6
2C19	Rifampin	10	20			
2D6	not identified					
2E1	not identified					
3A4	Rifampin	10-50	4-31	Phenobarbital Phenytoin Rifapentine Troglitazone Taxol Dexamethasone	100-2000 50 50 10-75 4 33-250	3-31 12.5 9.3 7 5.2 2.9- 6.9

Following treatment of hepatocytes for two or three days, the resulting enzyme activities can be determined using the appropriate CYP specific probe substrate list in Table III. The endpoint for induction prediction is to determine if the test article can produce a change that is equal or greater than 40% of what the positive inducer can produce.

$$\% \text{ positive control} = \frac{(\text{activity of test drug treated cells} - \text{activity of negative control}) \times 100}{(\text{activity of positive control} - \text{activity of negative control})}$$

Based on present knowledge, if a test article is not an inducer of CYP3A4, it can be concluded that it will not induce CYP2C8, CYP2C9 and CYP2C19. There are also other methods that can be used to evaluate induction including western blot, Real-Time RT-PCR and reporter gene assays (110).

CHAPTER 2

EFFECTS OF LYCOPENE ON PROTEIN EXPRESSION IN HUMAN PRIMARY PROSTATIC EPITHELIAL CELLS

2.1 Introduction

Prostate cancer ranks first in non-dermatological cancer incidence in men in the United States and is the second leading cause of cancer-related mortality in men and is anticipated 241,740 new cases and 28,170 deaths in 2012 (3). Prostate carcinogenesis is a multistep process induced by genetic and epigenetic changes that disrupt molecular pathways involved in cell proliferation, differentiation, apoptosis, and senescence (9). Precursor lesions in prostate tissue represent intermediate stages between normal and malignant epithelium (10). Since these lesions may appear 20 years before clinically evident cancer, the slow progression of prostate cancer might provide an opportunity for chemoprevention.

Lycopene is the most abundant carotenoid in tomatoes and tomato-derived products, and is the most potent antioxidant among the more than 600 naturally occurring carotenoids. Epidemiological studies in humans, animal models and controlled clinical trials indicate that blood lycopene levels and intake of tomato-derived products are inversely related to prostate cancer risk (112). Several clinical trials have shown that dietary tomato sauce or lycopene supplementation in men increases lycopene levels in prostate tissue and serum, reduces DNA oxidation in prostate tissue and lowers serum prostate specific antigen (PSA) (10, 45).

Postulated mechanisms of cancer chemoprevention by lycopene are shown in Figure 7. These mechanisms include inhibition of 5-lipoxygenase (63); regulation of the IGF-1/IGFBP-3 system (55-57); modulation of immune function (65); up-regulation of carcinogen-metabolizing enzymes and phase II protective enzymes (64, 66); inhibition of growth and induction of differentiation in cancer cells by modulating the expression of cell cycle regulatory proteins (54); prevention of oxidative DNA damage (35, 61); up-regulation of Cx43 and increased gap junction intercellular communication (58, 59); balancing redox signaling (60); and inhibition of inflammation and androgen receptor signaling (62). Evidence for these mechanisms of lycopene action have originated from a variety of experimental systems that have included cell culture, animal studies and clinical trials. However, these studies have usually focused on a particular pathway instead of looking at multiple mechanisms.

Previously, the van Breemen laboratory used iCAT quantitative proteomics to examine how lycopene affects protein expression in the LNCaP human prostate cancer cell line (113) and found that proteins involved in protecting cells from oxidative stress and detoxification of electrophilic compounds were up-regulated by lycopene. Here, we used a more sensitive iTRAQ proteomics approach to determine how cellular proteins are up or down-regulated upon exposure to lycopene. For the first time to our knowledge, the response to lycopene of normal human primary prostatic epithelial cells was investigated. The use of healthy prostate epithelial cells is important for prostate cancer chemoprevention studies since most prostate cancers originate in these basal epithelial cells (42, 114).

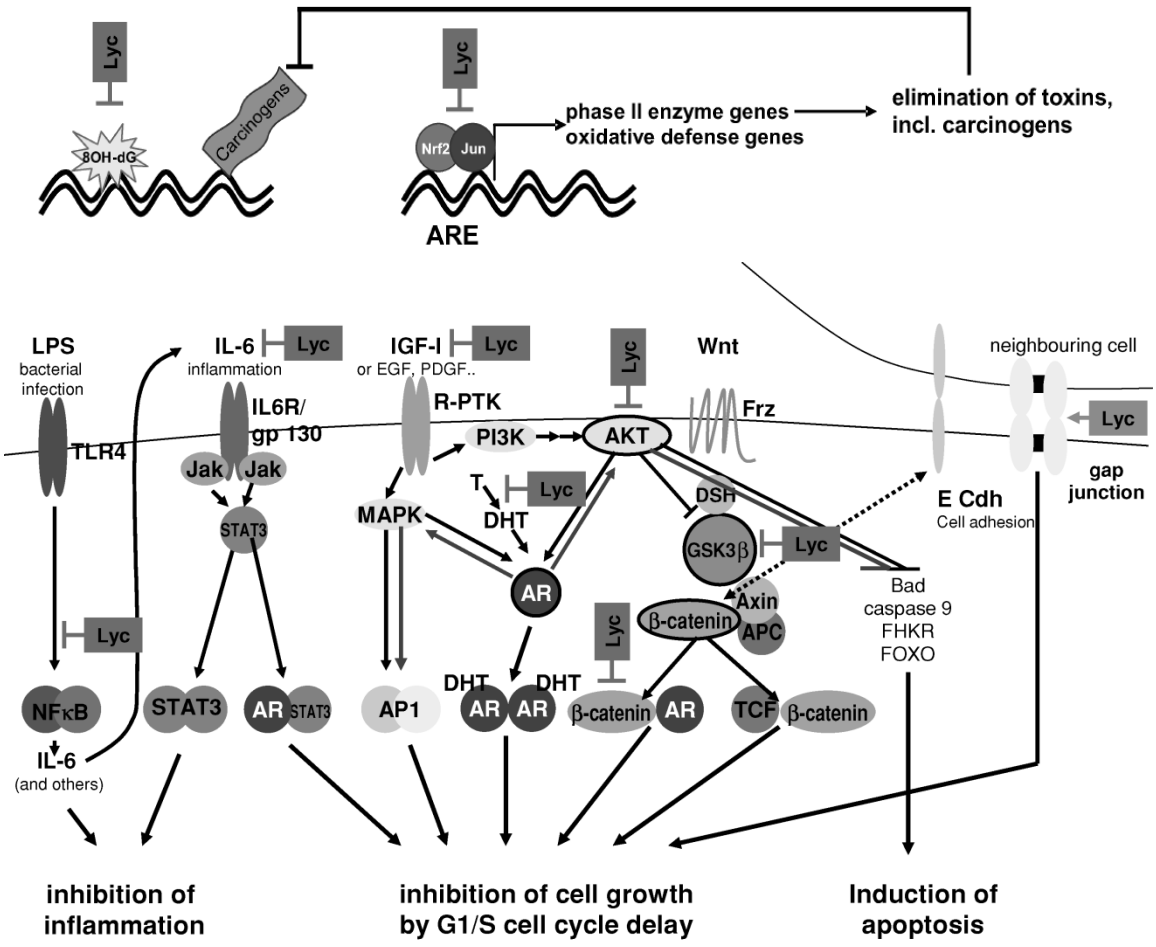


Figure 7. Postulated mechanisms of lycopene chemoprevention (115).

2.2 Materials and Methods

2.2.1 Materials

Cell culture medium PrEGM was purchased from Lonza (Walkersville, MD). Lycopene beadlets were gift from BASF. Protease inhibitor cocktail was purchased from Sigma (St. Louis, MO). iTRAQ reagents were purchased from AB Sciex (Foster city, CA), and trypsin was purchased from Promega (Madison, WI). The BioRad protein assay kit was purchased from Biorad (Hercules, CA). PBS buffer, all chemicals and HPLC-grade solvents were purchased from Thermo Fisher (San Jose, CA).

2.2.2 Human primary prostatic epithelial cell culture

Human primary prostatic epithelial cells were derived from radical prostatectomy specimens of patients who had received no prior chemical, hormonal or radiation therapy as described previously (116). Briefly, fresh prostate tissues were excised from the normal peripheral zone of the prostate, and the area was marked for histological analysis. The tissues were minced and digested overnight with collagenase. The digested tissues were plated onto collagen-coated dishes in PrEGM. When the cells reached 70% confluence, they were aliquoted into 25-50 freezer vials and cryopreserved in liquid nitrogen storage. Individual frozen aliquots of epithelial cells were thawed for each set of experiments, and cell cultures were incubated in humidified incubator with 95% air/5% CO_2 at 37°C in serum-free PrEGM. Cells were treated with 2 µM lycopene or placebo for 48 h after reaching 70% confluence.

2.2.3 Subcellular fractionation

After treatment with 2 µM lycopene or placebo, the cell culture medium was removed, and cells were washed twice with ice cold PBS buffer. Hypotonic buffer (200 µL) containing 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM DTT, 1 mM EDTA, and protease inhibitor cocktail 1% were added to the cells which were scraped into buffer and transfered to a new tube. Cells were disrupted by repeated freezing and thawing. The crude nuclear pellet was collected by centrifugation at 1,800 x *g* for 15 min at 4°C. The supernatant containing the cytosolic and membrane proteins were frozen at –80°C until use. The crude nuclear pellet was resuspended on ice in 0.5 volumes of low salt buffer containing 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 1 mM EDTA, and protease inhibitor cocktail 1%. While the nuclei were on ice, 0.5 nuclear volumes of high salt buffer containing 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1.2 M KCl, 1 mM DTT, 1 mM EDTA, and protease inhibitor cocktail 1% were added slowly to solubilize nuclear proteins. Triton-X100 (1%) was added to the suspension which was sonicated 4 times and centrifuged at 25,000 x *g* for 30 min at 4°C to pellet nuclear debris. The supernatant, which contained nuclear and nuclear membrane proteins, was stored at –80°C until use. The protein concentration of each cell fraction was determined by using the BioRad protein assay according to manufacturer's instruction.

2.2.4 Protein labeling by iTRAQ

Proteins from each fraction were digested by using trypsin and labeled with iTRAQ following the manufacturer's protocol with some modification. Briefly, 100 µg protein from each fraction was precipitated by acetone at –20°C for 2 h. Each protein pellet was dissolved in

0.5 M triethylammonium bicarbonate buffer with 0.1% SDS and reduced in 5 mM TCEP at 60 °C for 1 h. The reduced protein was then blocked in 10 mM MMTS by incubating at room temperature for 20 min and further digested at 37 °C overnight by trypsin (Promega, Madison, WI) with shaking. iTRAQ reagent in ethanol was added to each sample (> 60% ethanol in the reaction), and the reaction mixture was incubated at room temperature for 2 h. The reaction was stopped by adding an equal volume of water, and the experiment and control samples were mixed together.

2.2.5 Two dimensional microcapillary HPLC-tandem mass spectrometry

A PolySulfoethyl A SCX column (5 µm, 200 Å, 4.6 × 100 mm) from PolyLC (Columbia, MD) was used to fractionate digested iTRAQ labeled peptides. Mobile phase A consisted of 10 mM potassium phosphate (pH<3) and 25% acetonitrile, and mobile phase B consisted of 10 mM potassium phosphate (pH<3), 1 M KCl and 25% acetonitrile. Labeled peptides were diluted with 25% acetonitrile in water (pH<3) by at least 10-fold to reduce the concentration of buffer and iTRAQ reagents, loaded onto the SCX column and eluted as follows: 100% mobile phase A for 5 min, 0% to 10% mobile phase B over 5 min, 10% to 25% mobile phase B over 25 min, 25% to 50% mobile phase B for 10 min, 50% B for 5 min and return to 100% mobile phase A for 20 min. Fractions were collected every minute and combined according to UV 280 nm absorbance. The fractions were evaporated to dryness under nitrogen gas and reconstituted in 4% acetonitrile in water containing 0.1% formic acid immediately prior to LC-MS/MS analysis.

Labeled peptides were analyzed on a Thermo Finnigan (San Jose, CA) linear ion trap mass spectrometer (LTQ) equipped with a Dionex (Auburn, CA) microcapillary HPLC system.

Reverse phase HPLC was performed using an Agilent Zorbax 300SB C_{18} column (3.5 µm, 75 µm × 150 mm) and LC Packings C_{18} PepMap precolumn cartridge (5 µm, 0.3 mm × 5 mm). A step gradient was used consisting of a linear gradient from 5 to 55% solvent B over 120 min (solvent A: 95:5:0.1; and solvent B: 5:95:0.1, water/acetonitrile/formic acid, v/v/v) at a flow rate of 250 nL/min. Positive ion nanoelectrospray mass spectra were acquired in data-dependent mode in which each MS scan (*m/z* 400 to *m/z* 2000) was followed by four MS/MS scans using hybridization of pulsed Q dissociation (PQD) with a normalized collision energy of 31%, activation Q value of 0.6, activation time of 0.4 ms, and collision induced dissociation (CID) (117) with collision energy of 35%, activation Q of 0.25 and activation time of 30 ms. The four most abundant peptide ions in each mass spectrum except singly charged ions were dynamically selected to generate tandem mass spectra. Dynamic exclusion was used to prevent repetitive selection of the same ions for a period of 60 s.

The iTRAQ experiments were performed with three independent biological replicates, and each of the three biological experiments was analyzed three times by µ-LC/MS/MS as technical replicates to gather reliable quantitative information. Hybridization of PQD and CID was used to obtain reliable quantitation and sequencing of peptides ions.

2.2.6 Data analysis

The raw data from the MS/MS analysis were extracted automatically and converted to mzXML and MGF formats by using in house software. Two different search engines, MassMatrix (University of Illinois at Chicago, IL) (118, 119) and Mascot (Matrix Science, London, UK) were used to search against the International Protein Index (IPI.human.V3.5,

2008) with decoy option to improve confidence levels of protein quantitation and identification. The mass tolerance for precursor ions and fragment ions was set to 2 and 0.8 Da, respectively. Up to 2 missed trypsin cleavages were allowed. Variable modification was permitted to allow for the detection of methionine oxidation, and fixed modifications included β-methylthiolation of cysteine and the iTRAQ MS/MS tag of peptide *N*-terminals and lysine. Mascot searching was used to identify the protein hits, and the Mascot search results were further visualized and validated by Scaffold (Proteome Software, Portland, OR). MassMatrix was used to quantify the relative changes in protein expression and the individual protein expression ratio change was normalized by protein global expression ratio as described by Armenta *et al*. (120). For chemoprevention, we defined 10% change in protein expression as a significant change.

2.3 Results and Discussion

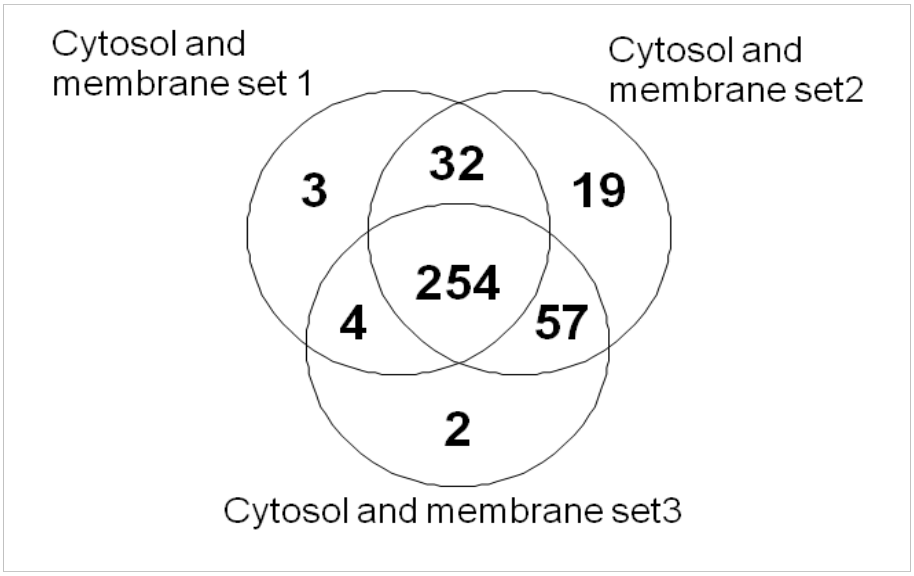
2.3.1 Proteomics of human primary prostatic epithelial cells

Human primary prostatic epithelial cells were grown in culture as a model of transit amplifying cells of the basal prostatic epithelium. These cells are androgen independent (114, 121) with high proliferation rate. They express basal and transitional cell type markers (43), cytokeratins 5 and 14, which was confirmed by our proteomics measurement. These basal cells have long been believed to be the cell origin for common prostatic adenocarcinomas, which has been confirmed by Goldstein *et al*. (42) using human primary prostate basal cells with lentivirus carrying genetic alteration genes in immunodeficient mice.

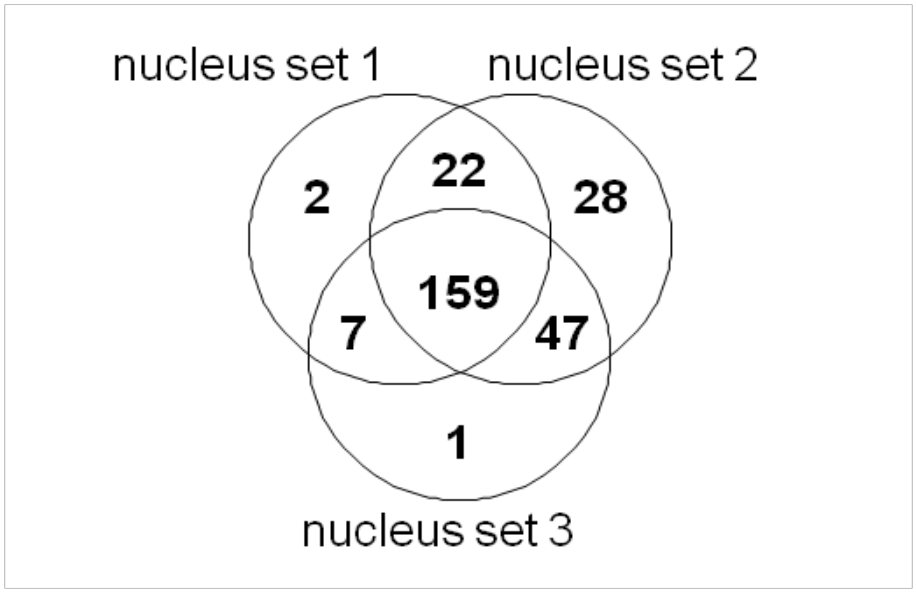
In the cytosolic and membrane fraction, a total of 371 proteins were identified including 254 proteins shared by all 3 experiments, 93 proteins identified in 2 experiments and 24 proteins

identified in just a single experiment (Figure 8A) with a false discovery rate of 0.0% protein and peptides. All the proteins identified have a probability score of at least 95% with at least 2 unique peptides, and the peptides probability score was over 90%. The gene ontology (GO) analysis of biological process of all the identified proteins in the cytosolic and membrane fraction is shown in (Figure 9A) and revealed that most are involved in metabolism, development, response to stimulus, and regulatory processes. The remaining are involved in immune, growth, multicellular and multiorganism processes. The GO analysis of molecular function (Figure 9B) indicated that most of the identified proteins have binding and catalytic activities and the remaining proteins have primarily antioxidant, transporter, translation regulator, enzyme regulator, carrier, and motor activities. Therefore, many proteins were identified in the cytosolic and membrane fraction that might be involved in mechanisms of action related to lycopene.

In the nuclear extract fraction, 266 proteins were identified in total including 159 proteins by all 3 experiments, 76 proteins in 2 experiments and 31 proteins identified in just one experiment (Figure 8B). The false discovery rate for protein and peptides was 0.8% and 0.1%, respectively. The GO analysis of biological process of all the identified proteins in the nuclear extracts is shown in Figure 10A and indicated that most were involved in metabolism, development, cell proliferation, cell growth, and regulatory processes. The other nuclear proteins that were identified were involved in communication, cell killing, immune response, and cell aging processes. The GO analysis of molecular function of nuclear proteins (Figure 10B) indicated that most have binding, catalytic and structural activities, followed by transporter, translation, transcription, and antioxidant function.

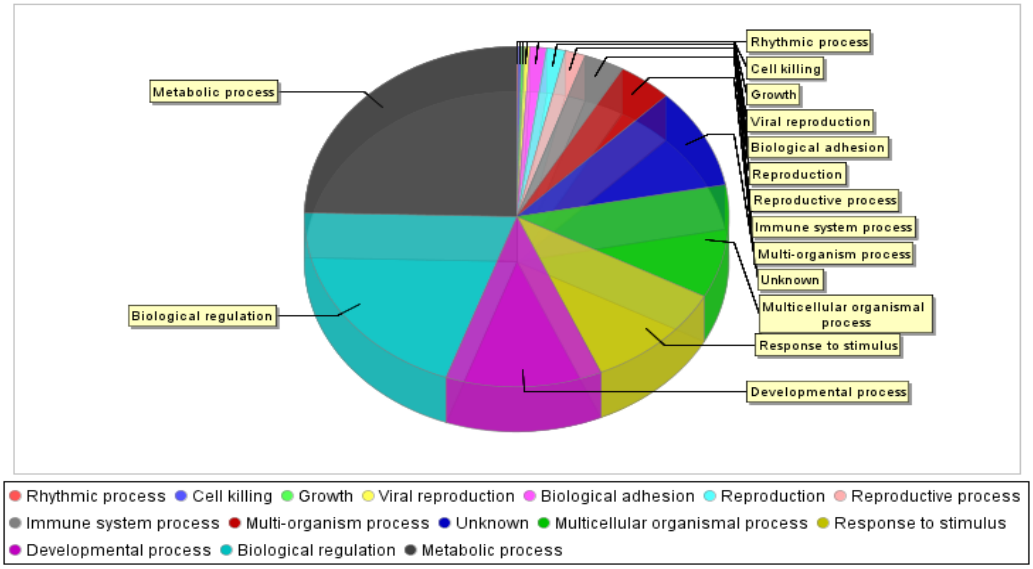


A

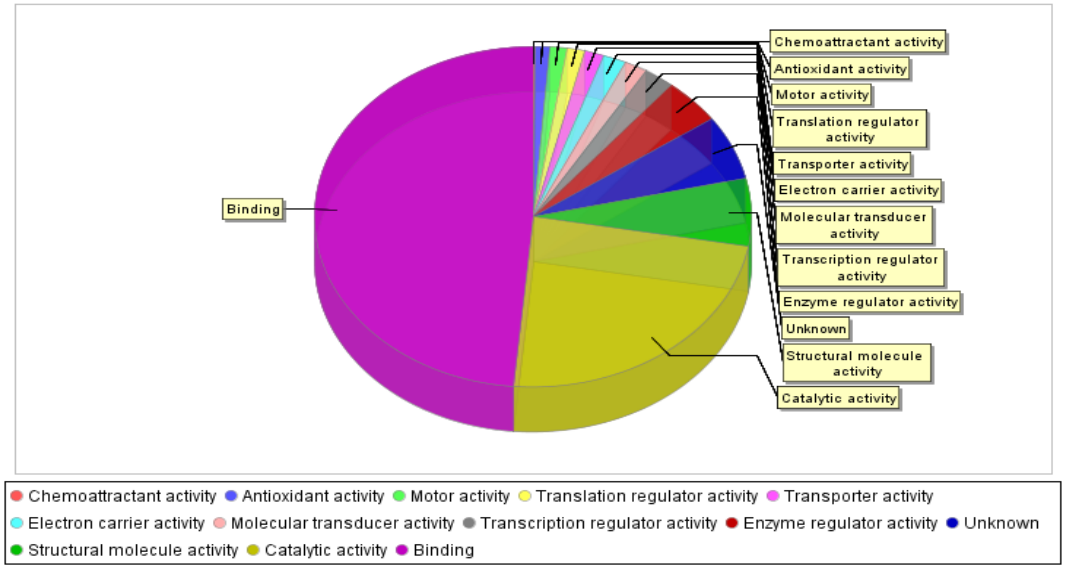


B

Figure 8. Venn diagram of proteins identified in the cytosol and nucleus from three independent biological experiments, (A) cytosol and membrane fraction, (B) nuclear fraction.

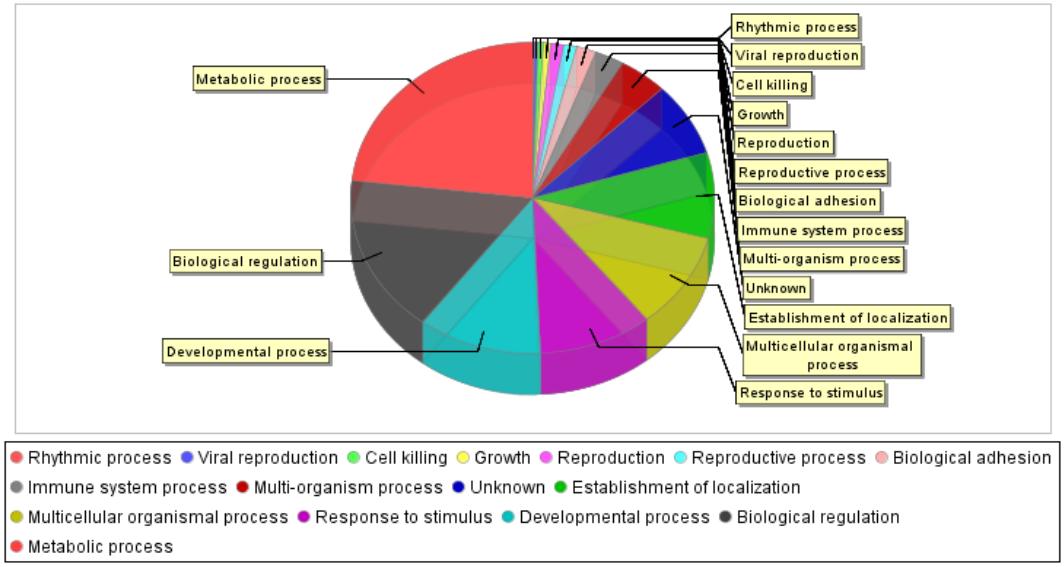


A



B

Figure 9. GO classification of identified proteins in cytosol and membrane based on (A) biological process; and (B) molecular function.



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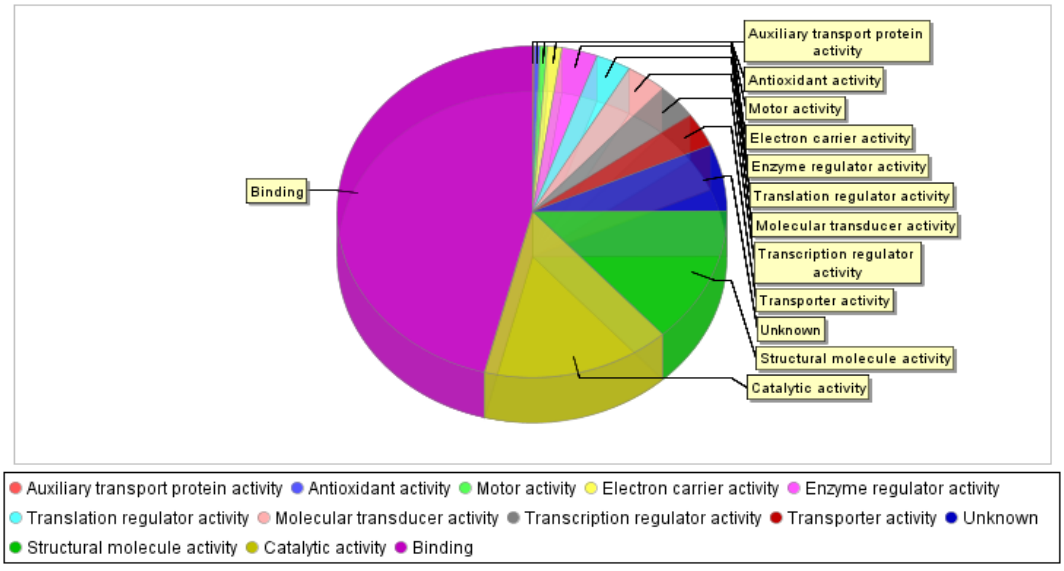


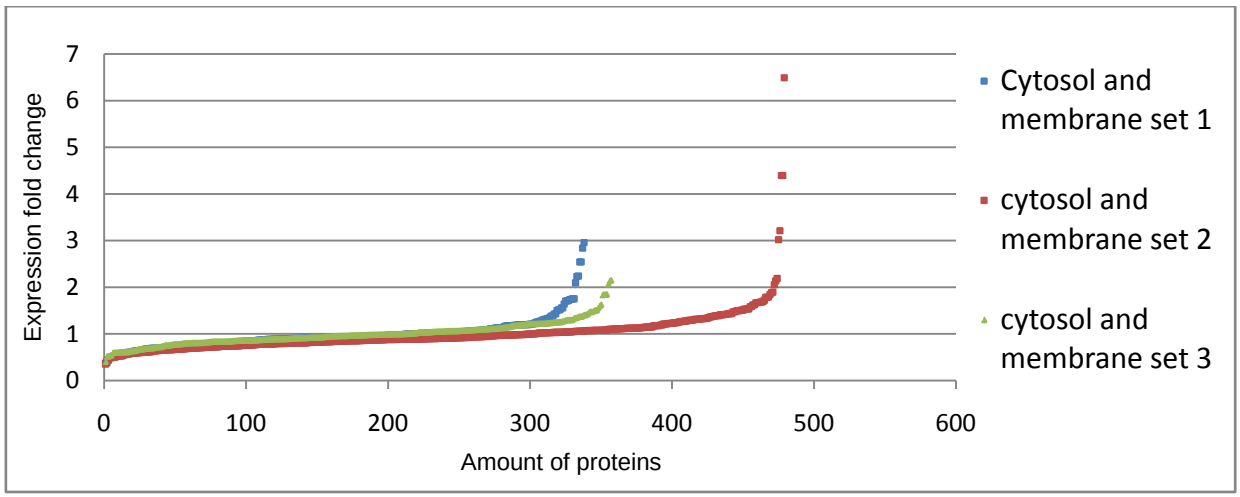
B

Figure 10. GO classification of identified proteins in the nucleus based on (A) biological process; and (B) molecular function.

2.3.2 Differential protein expression induced by lycopene

The protein expression level changes of the cytosolic and membrane fraction and the nuclear fraction were well distributed as shown in Figure 11 with average change close to 1.0. The distribution met the requirement of normalization by protein global expression ratio (120). A list of proteins modulated by lycopene treatment compared with placebo is shown in Table V and grouped according to their function in the cells. These proteins were identified and showed the same expression change in at least 2 experiments. As shown in Table V, lycopene treatment altered expression of several proteins with diverse molecular functions which included modulating cell redox homeostasis, apoptosis, cytoprotection, and androgen receptor signaling.

A representative spectrum of one peptide from Glutathione-S-transferase P1 (GSTP1) is shown in Figure 12. Figure 12A is the PQD and CID hybrid spectrum indicating all the detectable *b* ions and *y* ions of this peptide which give you the amino acid sequence of this peptide. Figure 12B is the low mass region showing the iTRAQ ion of *m/z* 145 and iTRAQ reporter ions of *m/z* 114 and *m/z* 116 from which the relative intensity of the peptide in different treatment condition was known. The database search engine will assign the peptide to specific protein and the protein relative expression change will be calculated based on this peptide and also all the other peptides assign to that protein.



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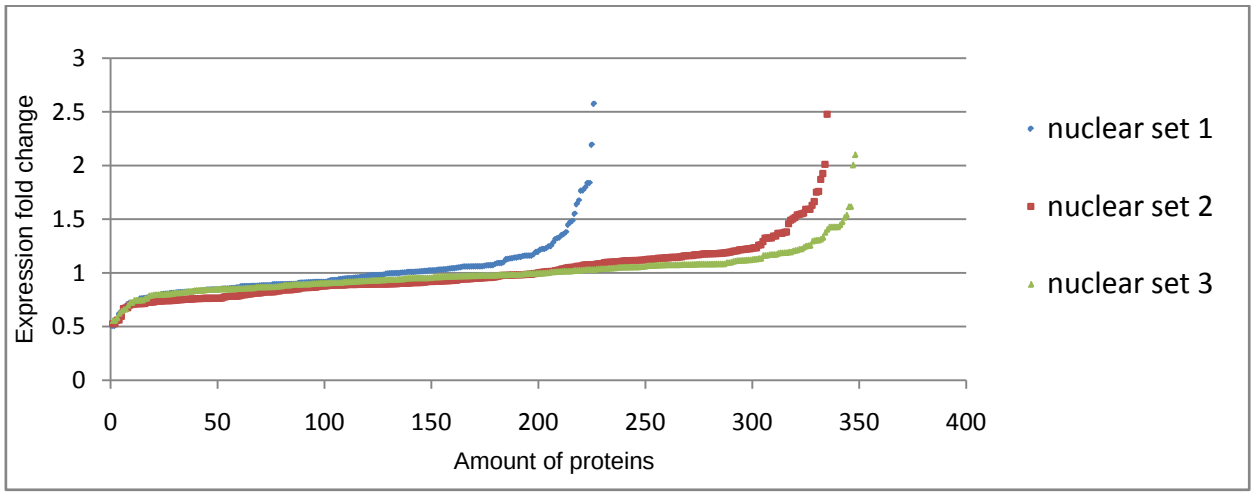


B

Figure 11. Three independent biological experiments quantitative results distribution, (A) cytosolic and membrane fraction, (B) nuclear fraction.

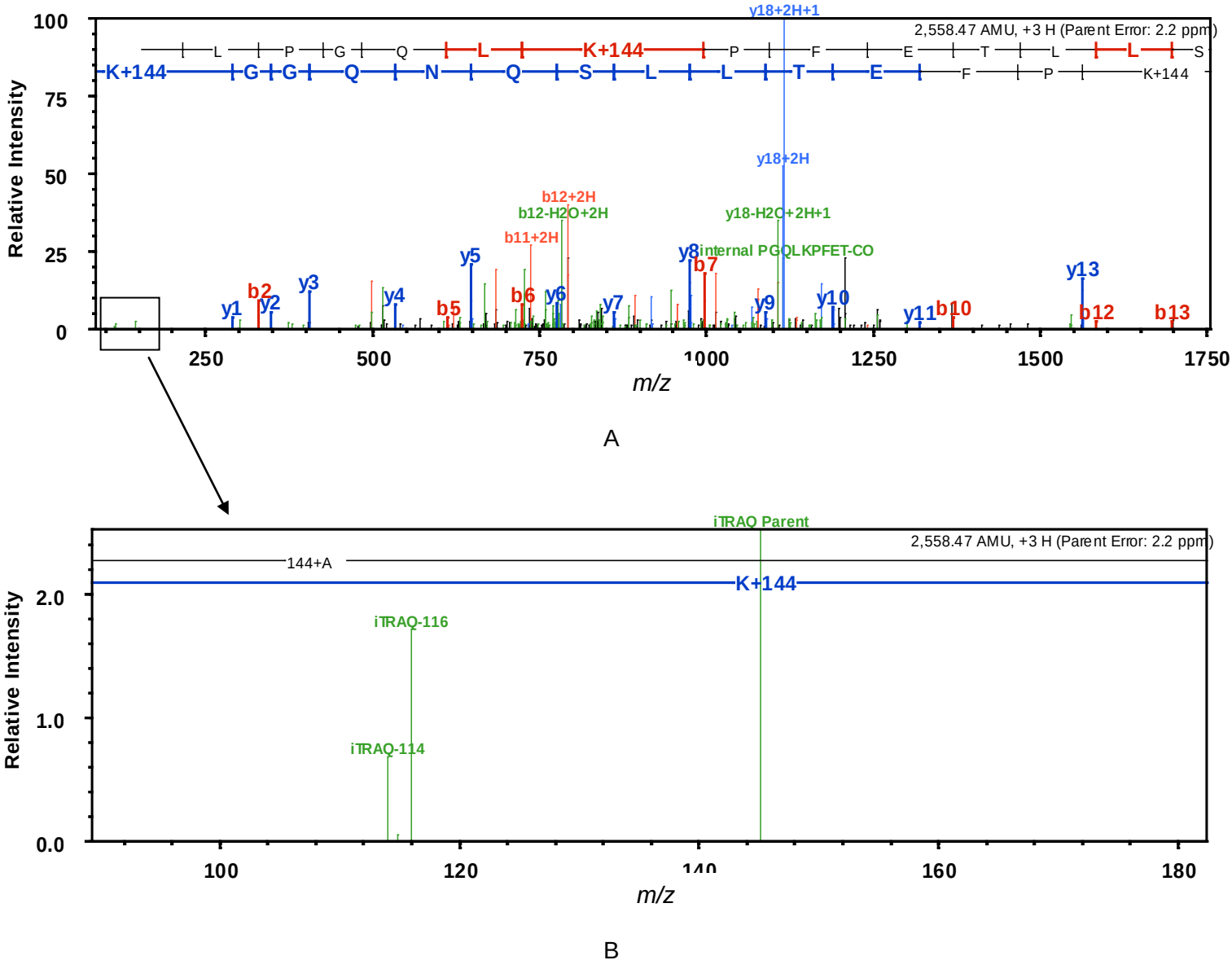


Figure 12. Positive ion nanoelectrospray mass spectrum of a peptide from GSTP1 protein which was up-regulated in the cytosolic and membrane fraction. The triply charged precursor ion was observed at *m/z* 853.8. (A) PQD and CID hybrid spectrum. (B) low mass region showing the iTRAQ reporter ions.

TABLE V

CHANGES IN EXPRESSION OF PROSTATE EPITHELIAL CELL PROTEINS DUE TO LYCOPENE TREATMENT

Accession number	Protein	Unique peptide	Fold expression change	Function
	Cytosol and membrane			
	Apoptosis			
IPI00021263	Protein kinase C inhibitor protein 1	7	0.85±0.13	Anti-apoptosis process
IPI00304925	Heat shock 70 kDa protein 1A/1B	6	0.88±0.10	Anti-apoptosis process
IPI00025512	Heat shock protein beta-1	4	0.90±0.04	Anti-apoptosis process
IPI00003815	Rho GDP-dissociation inhibitor 1	3	0.89±0.09	Anti-apoptosis process
IPI00550900	Translationally-controlled tumor protein	2	0.90±0.07	Anti-apoptosis process
IPI00220766	Lactoylglutathione lyase	4	0.87±0.01	Anti-apoptosis process
IPI00003362	78 kDa glucose-regulated protein	14	0.84±0.09	Anti-apoptosis process
IPI00007074	Tyrosyl-tRNA synthetase	3	1.20±0.09	Apoptosis
IPI00011253	40S ribosomal protein S3	6	1.15±0.07	Activation of caspase activity, induction of apoptosis
IPI00479186	Pyruvate kinase isozymes M2	19	1.15±0.07	Nuclear translocation induces cell death
IPI00010896	Chloride intracellular channel protein 1	6	0.65±0.05	Suppression of CLIC1 induce apoptosis and inhibit tumor growth
	Antioxidation & cytoprotection			
IPI00386755	ERO1-like protein alpha	10	0.80±0.06	Responsible for a significant proportion of reactive oxygen species (ROS)
IPI00642936	Glutathione S-transferase omega 1	4	1.11±0.05	Functions in the GSH-ascorbate cycle as part of antioxidant metabolism
IPI00009634	Sulfide:quinone oxidoreductase	8	1.13±0.06	Catalyzes the oxidation of hydrogen sulfide, with the help of a quinone
IPI00640741	Peroxiredoxin-1	7	1.14±0.09	Reduces hydrogen peroxide and alkyl hydroperoxides
IPI00219757	Glutathione S-transferase P 1	10	1.17±0.04	Conjugation of reduced GSH to exogenous and endogenous electrophiles
IPI00000877	Hypoxia up-regulated protein 1	4	1.56±0.19	Cytoprotective mechanisms triggered by oxygen deprivation
IPI00008223	UV excision repair protein RAD23 homolog B	3	1.49±0.35	Recognize various helix-distorting lesions in DNA and initiate global genome repair

TABLE V

CHANGES IN EXPRESSION OF PROSTATE EPITHELIAL CELL PROTEINS DUE TO LYCOPENE TREATMENT (continued)

Accession number	Protein	Unique peptide	Fold expression change	Function
	Signal transduction			
IPI00298547	Protein DJ1	4	0.77±0.13	Positive regulator of androgen receptor-dependent transcription
IPI00027230	Heat shock protein 90 kDa beta 1	9	0.89±0.08	AR chaperon protein which stabilize AR in the cytosol
IPI00646689	Thioredoxin domain-containing protein 17	3	0.87±0.07	Deficiency enhance TNF-alpha induced apoptosis
IPI00293276	Macrophage migration inhibitory factor	2	0.76±0.04	A proinflammatory cytokine, positive regulation of ERK1 and ERK2 cascade
IPI00022078	Protein NDRG1	4	1.42±0.21	Act as tumor suppressor in many cell types
IPI00411765	Epithelial cell marker protein 1	7	0.88±0.08	Regulates protein synthesis and epithelial cell growth by stimulating Akt/mTOR pathway
		Nucleus		
IPI00411765	Epithelial cell marker protein 1	7	0.84±0.08	Regulates protein synthesis and epithelial cell growth by stimulating Akt/mTOR pathway
IPI00021405	Prelamin-A/C	7	0.84±0.14	It acts to disrupt mitosis and induce DNA damage

Apoptosis

Lycopene treatment of human primary prostatic epithelial cells up-regulated proteins associated with apoptosis induction and down-regulated proteins involved in anti-apoptosis processes (Table V). The proteins associated with apoptosis induction were tyrosyl-tRNA synthetase (TyrRS) which was upregulated by 20%, 40S ribosomal protein S3 (RPS3) upregulated by 15%, pyruvate kinase isozyme M2 (PKM2) upregulated by 15% and chloride intracellular channel protein 1 (CLIC1) which was downregulated by 35%. TyrRS has been reported by Wakasugi *et al*. to be produced by cells under conditions that induce cell death by apoptosis (122). RPS3 can induce apoptosis by activating caspase, while PKM2 plays a general role in caspase independent cell death of tumor cells. Suppression of CLIC1 protein can induce apoptosis, enhance TNFα induced apoptosis and inhibit tumor growth as reported by Suh *et al*. (123). All of these effects indicate that lycopene enhances apoptosis signaling in primary prostate epithelial cells.

The proteins down-regulated by lycopene treatment including heat shock 70 kDa protein (HSP70) 1A/1B (down by 12%), heat shock protein beta 1 (HSP27) (down by 10%), Rho GDP-dissociation inhibitor 1 (Rho GDI 1) (down by 11%), translationally-controlled tumor protein (TCTP) (down by 10%), lactoylglutathione lyase (down by 13%), 78 kDa glucose-regulated protein (Grp78) (down by 16%), and protein kinase C inhibitor protein 1 (KCIP1) (down by 15%). HSP70 directly inhibits apoptosis by blocking the recruitment of procaspase-9 to the Apaf-1/dATP/cytochrome c apoptosome complex (124). HSP27 is an important chaperon protein which protects cancer cells against apoptosis by interacting with the outer mitochondrial membranes and interfering with the activation of

cytochrome/c/Apaf-1/dATP complex to inhibit the activation of procaspase-9 (125). Zhang *et al.* reported that Rho GDI 1 protects cancer cells against drug-induced apoptosis (126). TCTP is stabilized by anti-apoptotic protein myeloid cell leukemia sequence by Zhang *et al*. (127), and its level is down-regulated through activation of the tumor suppressor protein p53 (128). Grp78 expression is associated with the development of castration resistance prostate cancer (129). KCIP1 is known to bind to MEKK1, which is activated by caspase 3, to block apoptosis (130). The down-regulation of all these proteins involved in anti-apoptosis processes indicates that lycopene enhances the apoptosis signal in primary human prostate cells.

This strong apoptotic activity is consistent with Ivanov *et al*. and Hwang *et al*. who reported apoptosis induction in prostate cancer cells by lycopene (67, 131) and Bowen *et al*. and Kim *et al*. who found that lycopene increased the apoptotic index in hyperplastic and neoplastic cells in human prostate BPH and cancerous tissue (53, 132). Overall, our results indicate that lycopene can induce apoptosis in the prostate epithelial cells by down regulating expression of anti-apoptotic chaperon proteins, activating caspase activity and promoting the caspase independent cell death pathway.

cytoprotection and redox homeostasis

GSTP1, which conjugates exogenous and endogenous electrophiles with reduced GSH, was up-regulated by 17%. Silencing of GSTP1 by promoter hypermethylation is found frequently in prostate cancer and has also been detected in proliferative inflammatory atrophy lesions (133, 134). Glutathione-S-transferase Omega 1 (GSTO1), which functions in the GSH-ascorbate cycle to detoxify hydrogen peroxide, was up regulated by 11%.

Peroxiredoxin 1, an antioxidant enzyme that reduces hydrogen peroxide and alkyl hydroperoxides, was up-regulated by 14%, and sulfide-quinone oxidoreductase, which catalyzes the oxidation of hydrogen sulfide, was up-regulated by 13% in prostate epithelial cells treated with lycopene. These effects indicate that another function of lycopene is to increase levels of phase II protective enzymes that can prevent cytotoxicity due to xenobiotic electrophiles and carcinogens. Upregulation of phase II enzymes is involved in detoxifying and eliminating toxic agents including carcinogens.

Hypoxia up-regulated protein 1, which has a pivotal role in cytoprotective cellular mechanisms triggered by oxygen deprivation, was up regulated by 56% in prostate epithelial cells treated with lycopene. The UV excision repair protein RAD23 homolog B, which plays a central role in DNA repair, was up-regulated by 49%. Both of these effects indicate that lycopene stimulates cytoprotective functions in prostate epithelial cells that can help prevent cancer initiation.

Lycopene down-regulated ERO1-like protein alpha by 20%, which is a cytosolic protein responsible for a significant proportion of reactive oxygen species (ROS) in cells. Also, CLIC1 which is usually expressed during oxidative stress, was down regulated by 35%. These results show that lycopene not only can function as an antioxidant but also can reduce ROS generation in the cell. These results are consistent with reports that lycopene reduces oxidative DNA damage in cell culture (135), in rat prostate tissue (136) and in human prostate tissue (47).

Signal transduction interference

Lycopene has been reported to alter signaling in the prostate including inflammation signaling, growth factor signaling, and steroid hormone signaling (115). Lycopene treatment of prostate epithelial cells down-regulated Thioredoxin domain-containing protein 17 (TXNDC17) by 13%. Deficiency of TXNDC17 enhances tumor necrosis factor alpha (TNFα) induced activation of caspase and subsequent apoptosis (137). Macrophage migration inhibitory factor (MIF), which is proinflammatory cytokine and a positive regulator of the MAPK pathway, was down-regulated by 24%. The effect can reduce inflammation to the cell, inhibit cell proliferation and slow down prostate cancer progression to androgen-independent growth (112) (71).

Protein DJ1, a positive regulator of androgen receptor dependent transcription, was down regulated by 23%, indicating that the androgen regulated transcription level would be reduced and androgen targeted genes transcription would be repressed. HSP90, an AR chaperon protein that helps to stabilize AR in the cytosol, was down regulated by 11%. Inhibitors of HSP90 cause AR degradation and are currently in clinical trials for prostate cancer therapy (138). These results are consistent with Herzog A *et al*. (68) and Siler U *et al*. (62), who found that lycopene reduced androgen signaling and AR target gene expression including prostatic steroid binding protein C1 and C3, cystatin related protein 2, and seminal vesicle secretion protein IV. Down-regulation of androgen signaling by lycopene should contribute to decreased androgen-dependent prostate growth.

Protein NDGR1, which is a tumor suppressor in many cell types and is necessary for the p53/TP53 mediated caspase activation and apoptosis, was up regulated by 42% in prostate epithelial cells treated with lycopene. Epithelial cell marker protein 1 (SFN), which regulates

protein synthesis and epithelial cell growth by stimulating the Akt/mTOR pathway, was down-regulated by 12%. The PI3K/AKT/mTOR pathway has been extensively studied and is considered to be a target for prostate cancer therapy (9). Reduced AKT signaling by lycopene might prevent the progression of prostate cancer to an androgen-independent state. These observations are consistent with lycopene growth inhibitory effects reported by other groups studying prostate cancer cells and normal prostate epithelial cells (67, 131, 139). Repression of the AKT/mTOR pathway by down-regulation of SFN would contribute further to chemoprevention by lycopene by inhibiting prostate cancer progression to an androgen independent state.

Compared with cytosolic and membrane proteins, the expression of fewer nuclear proteins was affected by lycopene. Prelamin-A/C, which disrupts mitosis and induces DNA damage, was down regulated by 16%. Down regulated by 12% in the cytosol, SFN was also down regulated in the nucleus by 16%. Since the AKT/mTOR pathway has been estimated to be up-regulated in 30-50% of prostate cancer (9), down-regulation of SFN should also result in down-regulation of AKT/mTOR and help prevent prostate cancer promotion and progression.

2.4 Conclusions

Prostate cancer initiation, promotion and progression involves multiple molecular pathways connected through cross-talk. Investigation of changes in the level of one or only a few proteins due to lycopene exposure cannot detect pleiotropic effects. Our proteomics approach shows how lycopene exerts multiple protective effects on human prostatic epithelial cells. Several proteins were up or down-regulated by lycopene that can reduce oxidative stress in the cell. Up

regulation of phase II enzymes such as GSTP1, which is often silenced in prostate cancer, and GSTO1 and SQR can help prevent cancer initiation by detoxifying potentially carcinogenic electrophiles. Lycopene was found to inhibit proliferation of prostate epithelial cells by down regulating the AKT/mTOR pathway and by up regulating genes that have growth inhibitory effects. Lycopene was shown to induce caspase dependant apoptosis and down regulate several proteins involved in anti-apoptosis process. Lycopene was also found to alter several signaling pathways, including inhibition of androgen signaling, down regulating TNFα signaling, deactivating MAPK pathway, reduce inflammation, and down regulation of the AKT/mTOR pathway to slow down proliferation and induce apoptosis. All of these lycopene effects on cellular proteins contribute to the prevention of cancer initiation, promotion, and progression. Our results showed that lycopene is able to modulate all of these chemoprevention pathways, providing a promising rationale for prostate cancer risk reduction.

CHAPTER 3

INDUCTION STUDIES OF CYP1A2 AND CYP3A4 IN HUMAN HEPATOCYTES BY A HOP EXTRACT

3.1 Introduction

The female flowers of hops (*Humulus lupulus* L.) which are rich in polyphenolic compounds are widely used in the brewing industry to preserve beer and give beer the signature flavor. Hops has a long history of use as a medicinal herb to treat a wide range of disorders and including use as a mild sedative, but the research on the clinical effectiveness and possible active constituent(s) remains inconclusive (76). Recently, the estrogenic properties and the potential cancer chemopreventive activities of hop extracts have been investigated, because dietary supplements containing hop extract are being used by menopausal women seeking alternatives to hormone replacement therapy. Some estrogenic compounds from hops have been intensively studied. Among them, 8-prenylnaringenin (8-PN) is considered to be a potent phytoestrogen, which binds to estrogen receptor (ER) and preferentially to ERα (102, 103, 107, 140, 141), while xanthohumol (XN) is believed to have cancer chemoprevention activity by inhibiting the metabolic activation of procarcinogens, inducing phase II detoxifying enzymes, and inhibiting tumor growth at early stages (78, 85, 90) . In addition, isoxanthohumol (IX), which is at least 10-fold more abundant than 8-PN in most hop products, functions as a precursor to 8-PN as it can be O-demethyated by cytochrome P450 enzymes and/or by intestinal micro biota (142, 143). Therefore, the estrogenic potency and potential health effects of hop derived products not only depend on levels of 8-PN, but also on the amount of IX.

Metabolism of the major prenylated flavonoids of hops has have been investigated by several research groups. Nikolic *et al*. reported hydroxylation on the prenyl methyl group of IX to be the major route of oxidative metabolism forming either cis or trans hydroxylated metabolites but only the trans isomer of XN (144). 8-PN, the potent phytoestrogen in hops, was identified as a demethylation product of IX. Twelve other oxidative metabolites were formed from 8-PN incubated with human liver microsomes. The major site of oxidation was also shown to be on the prenyl methyl group (140). Other than phase I oxidative metabolites, phase II glucuronic acid conjugates of IX and 8-PN were also found by several research groups using *in vitro* models as well as *in vivo* (144-148).

Besides these metabolite identification studies, specific human CYP isoforms responsible for the biotranformation of IX to 8-PN have been identified. CYP1A2 was found to catalyze the *O*-demethylation of IX to generate 8-PN. Moreover, CYP2C19 was responsible for the oxidation on the prenyl side chain of 8-PN (149)**.**

Little is known about the interactions of hops and human metabolizing enzymes. Recently, Hemachandra *et al*. reported that estrogen-induced expression of CYP1B1 and CYP1A1 was attenuated by a hop extract and that 8-PN was found to be a potent inhibitor of CYP1A1 and 1B1 (99). Several other studies have also confirmed that 8-PN can inhibit CYP1A2 (88). However, there are few reports on the potential for hops to induce cytochrome P450 enzymes. Induction of CYP enzymes can lead to adverse drug interactions by increasing the metabolism of other drugs that are substrates for the induced isoform. Due to the possibility of DDI resulting in toxicity, FDA encourages *in vitro* assessment of each new chemical entity (NCE) as conclusions from these studies may lead to safer and more efficient clinical trials. Here we

report a rapid *in vitro* activity assay utilizing cryopreserved human hepatocytes and ultra high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS-MS) together with real-time RT-PCR to evaluate the potential of cytochrome P450s induction by a standardized hop extract.

3.2 Materials and methods

3.2.1 Chemicals and reagents

All organic solvents were HPLC grade or better and were purchased from Thermo Fisher (Hanover Park, IL). Purified water was prepared by using a Millipore Milli-Q purification system (Millipore, Billerica, MA). Hanks Balanced Salt Solution (HBSS), pH 7.4, rifampin, omeprazole, midazolam, phenacetin, flurazepam, caffeine, and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO). 1'-Hydroxymidazolam was purchased from Cerilliant (Round Rock, TX). CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI). A standardized hop clinical extract was provided by the UIC/NIH Center for Botanical Dietary Supplements Research (Chicago, IL). Quantitative LC-MS-MS analysis using authentic reference compounds was used to determine that this hop extract contained 33.84% XN, 0.35% 8-PN, 1.77% 6-PN, and 1.07% IX (w/w).

Cryopreserved human hepatocytes, recovery media, culture media, fetal bovine serum (FBS), and 24-well culture plates coated with collagen I were obtained from BD Biosciences (San Jose, CA). See appendix for all the donors information.

3.2.2 Culture of cryopreserved human hepatocytes

Cryopreserved hepatocytes were thawed in a 37 °C water bath for 2 min with gentle shaking, then quickly poured into the recovery media and gently inverted 2-3 times before centrifuging at 100 *g* for 10 min at room temperature. The supernatant containing nonviable cells was carefully removed by aspiration and the plating medium containing 10% FBS was added to the resulting pellet. The pellet was dispersed, and the cell number was determined using a hemocytometer after staining with trypan blue. Hepatocytes (0.4 million cells per well) were plated in 24-well culture plates, and cells were incubated at 37°C and 95% humidity with 5% CO_2 for 3-4 h before the plating medium was replaced with culture medium. The culture medium was changed daily for 2 days. Following the equilibration stage, hepatocytes were incubated with culture media containing 50 µM omeprazole (CYP1A2 inducer), 10 µM rifampin (CYP3A4 inducer), 0.1% DMSO as a negative control, or one of four concentrations of a hop clinical extract (80, 400, 2000, and 5000 ng/mL). The induction medium was replaced daily, and mRNA measurements was carried out after two day, and enzyme activity assays were carried out after three days. CYP1A2 and CYP3A4 enzyme activities were determined independently in separate plates, and the experiments were carried out in triplicate for assessment of both CYP1A2 and CYP3A4.

3.2.3 Morphological cytotoxicity assessment

The cell cultures were inspected using a Nikon research microscope equipped with phase-contrast optics, a 3-CCD camera, and imaging computer with image analysis software. Inspections were performed daily after treatment with the hop extract or DMSO control.

Alterations in hepatocyte morphology such as changes in cell shape, loss of cell-cell contact, and accumulation of vacuoles would indicate cytotoxicity and possibly result in loss of enzyme activity (150).

3.2.4 CYP1A2 and CYP3A4 enzyme activity measurements by LC-MS-MS

After treatment with positive inducers and hop extract, hepatocytes were washed twice with HBSS. Phenacetin (50 µM) and midazolam (10 µM), substrates for CYP1A2 and CYP3A4, respectively, were added to the hepatocyte cultures and incubated at 37°C with shaking for 30 min. Medium was harvested and analyzed using UHPLC-MS-MS to determine enzymatic products of the probe substrates. Fresh pre-warmed medium was added to the intact monolayer for assessment of cell viability.

Aliquots (100 µL) of the harvested cell culture medium were mixed with an equal volume of acetonitrile containing the internal standards caffeine and flurazepam (250 ng/mL final concentration), vortexed for 1 min, and centrifuged at 8000 *g* at 4 °C for 10 min. A 100 µL aliquot of each supernatant was diluted with 100 µL water and then analyzed using UHPLC-MS-MS. For each analysis, 5 µL of sample was injected onto the column. UHPLC-MS-MS analysis was carried out using a Shimadzu LC-MS 8030 (Kyoto, Japan) triple-quadrupole mass spectrometer equipped with a Shimadzu LC-30AD Nexera UHPLC system. Chromatographic separation was carried out using a Shimadzu Shimpak XR-ODS III (2.1 x 50 mm, 1.6 µm) C_{18} column. During UHPLC-MS-MS, the following SRM transition were monitored: *m/z* 152→110 (acetaminophen), *m/z* 195→138 (caffeine), *m/z* 388→315 (flurazepam), and *m/z* 326→291 (1'-OH-midazolam). The mass spectrometer source

parameters are as follows: DL temperature was 300°C, the spray voltage was 3500 V, the nebulizing gas flow was 3 L/min, and drying gas flow was 20 L/min. The mobile phase consisted of a linear gradient from 20-100% methanol in 0.1% aqueous formic acid at a flow rate of 0.5 mL/min. A weighting factor of 1/x was applied to the linear regression for quantitation of metabolites. Enzyme activity was expressed as pmol of metabolite/min/10^6 cells plated.

3.2.5 RNA isolation and real-time RT-PCR analysis of enzyme mRNA

Total mRNA was extracted using the RNeasy mini kit (Qiagen, CA). Briefly, cells were washed twice with cold PBS (pH 7.4) and then lysed by adding 350 µL RLT buffer with 1% β-mercaptoethanol. Cells were then collected by rubber policeman and transferred to microcentrifuge tubes. Each homogenized cell lysate were passed through a Qiagen QIAshredder spin column, 350 µL 70% ethanol was added, mixed well, and the mixture was transferred to a RNeasy spin column. The lysate was washed with RW1 buffer once and RPE buffer twice. Total mRNA was eluted by 30 µL RNase-free water, and the mRNA concentration was measured by using Nanodrop (Thermo, CA).

Equal amounts of mRNA from each sample were used for reverse transcriptase PCR (RT-PCR) by using a SuperScript III cDNA Synthesis kit (Invitrogen, CA). Briefly, mRNA was mixed with 1 µL of 50 uM oligo(dT)20, 1 µL of 10 mM dNTP mix and an appropriate amount of DEPC-treated water to a final volume of 10 µL. The mixture was incubated at 65°C for 5 min and placed on ice for at least 1 min. Then, 10 µL of cDNA synthesis mixture containing 10x RT buffer, $MgCl_2$, DTT, RNaseOUT, and Superscript III reverse transcriptase was added and

incubated at 50°C for 50 min, 85°C for 5 min. Finally, 1 µL of RNase H was added and incubated at 37°C for 20 min. A 1 µL aliquot of the RT-PCR generated cDNA from each sample was mixed with 10 µL 2X gene expression assay PCR master mix, 1 µL of 20X TaqMan gene expression assay primers/probe from Applied Biosystem inventory (CYP3A4 assay ID Hs00430021_m1, CYP1A2 assay ID Hs01070374_m1), and 8 µL of DEPC treated water. GADPH (assay ID Hs99999905_m1) was used as an endogenous control gene. Gene amplification was carried out for 2 h using a Step-One™ real-time PCR system (Applied Biosystem, CA).

3.2.6 Assessment of cell viability

Following the assessment of enzyme activity, the cell monolayer was assessed for viability using the CellTiter 96 AQueous One Solution Cell Proliferation Assay following the manufacturer's protocol. Blank medium containing no cells was used as a negative control. Briefly, the MTS reagent was diluted 1:5 into fresh pre-warmed medium, and 180 µL was dispensed into each well. The plates were incubated at 37°C in 5% CO_2 for 45 min, and medium/MTS reagent was transferred to a clear bottom 96-well plate for spectrophotometric analysis at 490 nm.

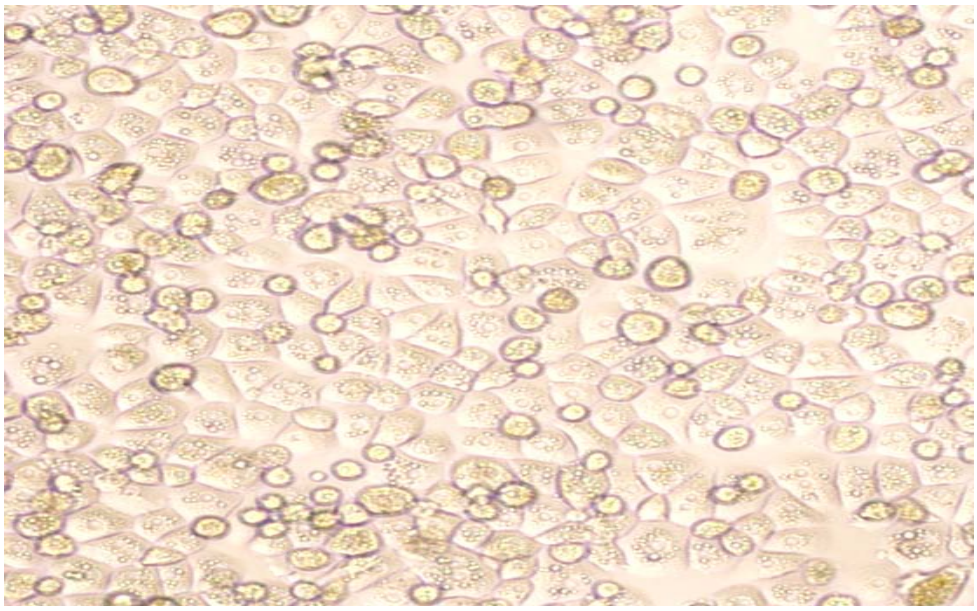
3.2.7 Statistical analysis

All of the experiments were carried out in triplicate. All data were expressed as the mean ± SD. The statistical analysis of these results consisted of t-test or ANOVA using GraphPad Prism 5.0.

3.3 Results

3.3.1 Morphology assessment

Cell morphology was examined daily during treatment with the hop extract to evaluate cytotoxicity. As shown in Figure 13 ,compared with control cultures, loss of cuboidal shape, accumulation of vacuoles and loss of cell cell contact were not observed in any hepatocyte cultures treated with hops extract at all concentrations . Cells were tolerant of the hop extract at the concentrations tested, and there were no visual differences between hop treated and the DMSO treated cells upon microscopic evaluation.



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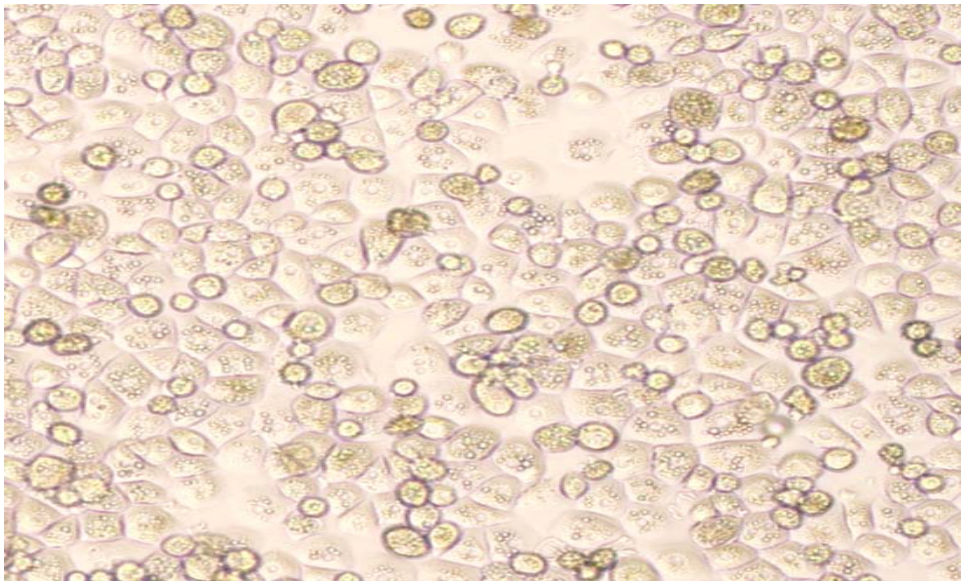


B

Figure 13. Morphology of human hepatocytes treated with hop extract (A) and with 0.1% DMSO control (B).

3.3.2 Enzyme activity and qRT-PCR

The UHPLC separation of the two CYP substrates, their corresponding metabolites and the two internal standards was carried out in less than 2 min with a total run time less than 3 min as shown in Figure 14. The linear ranges of the calibration curves for the metabolites were from 0.1 to 100 ng/mL for acetaminophen (CYP1A2) and 0.01 to 10 ng/mL for 1'-OH-midazolam (CYP3A4). The standard curves for both metabolites are shown in Figure 15 and Figure 16. These linearity of both the standard curves was determined using linear regression with 1/x weighing and the r^2 was 0.998.

The basal inducible potential of cryopreserved human hepatocytes from three different donors was evaluated, and the results are summarized in Figure 17 and Figure 18. All three donors showed good inducible potential that correlated well with the supplier's characterization data. Omeprazole induced CYP1A2 enzyme activity 32, 25 and 27-fold and mRNA levels over 125, 53 and 76-fold compared with the negative control (0.1% DMSO). Rifampin treatment induced CYP3A4 enzyme activity 19, 28 and 23-fold and mRNA 170, 360 and 450-fold compared with the negative control. Note that the changes in mRNA levels were consistent with the induction in enzyme activities in response to prototypical CYP1A and CYP 3A type inducers.

Four concentrations of the standardized hop extract (80 ng/mL to 5000 ng/mL) were tested for induction of mRNAs and CYP1A2 and CYP3A4 activities. The selection of this concentration range was based on the measurement of ~30 ng/mL total XN in serum concentration during the phase I clinical trial of a hop extract at the UIC/NIH Center for Botanical Dietary Supplement Research (data not shown), and that the amount of XN in the

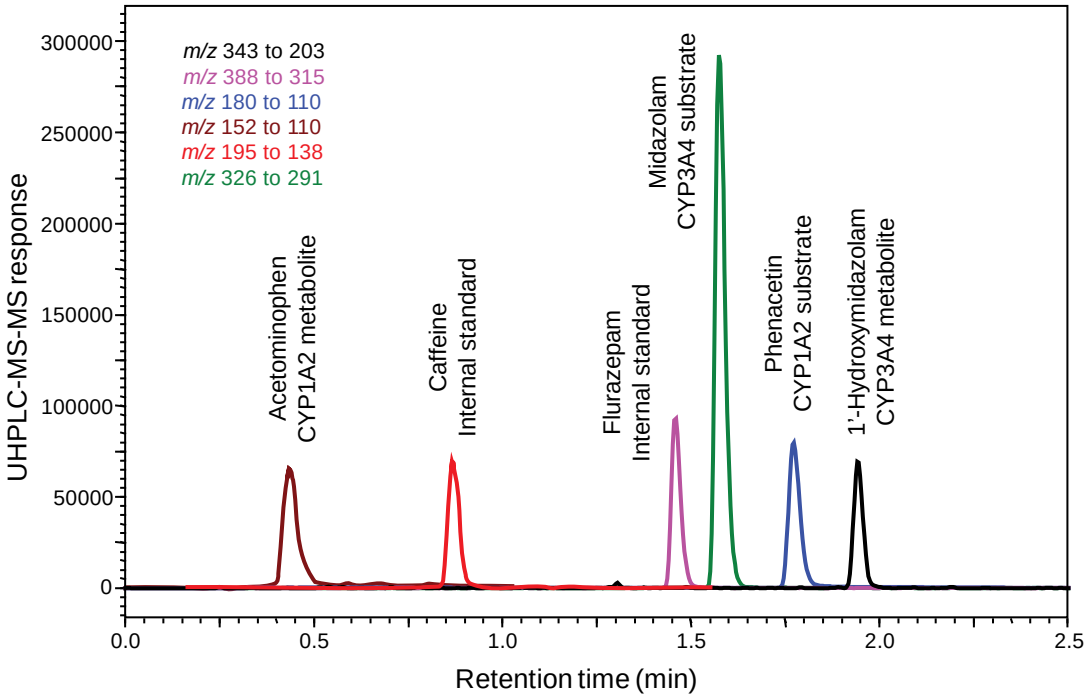


Figure 14. Positive ion electrospray UHPLC-MS-MS chromatograms of cytochrome P450 substrates, their corresponding metabolites and internal standards. The separation was carried out within 2 min.

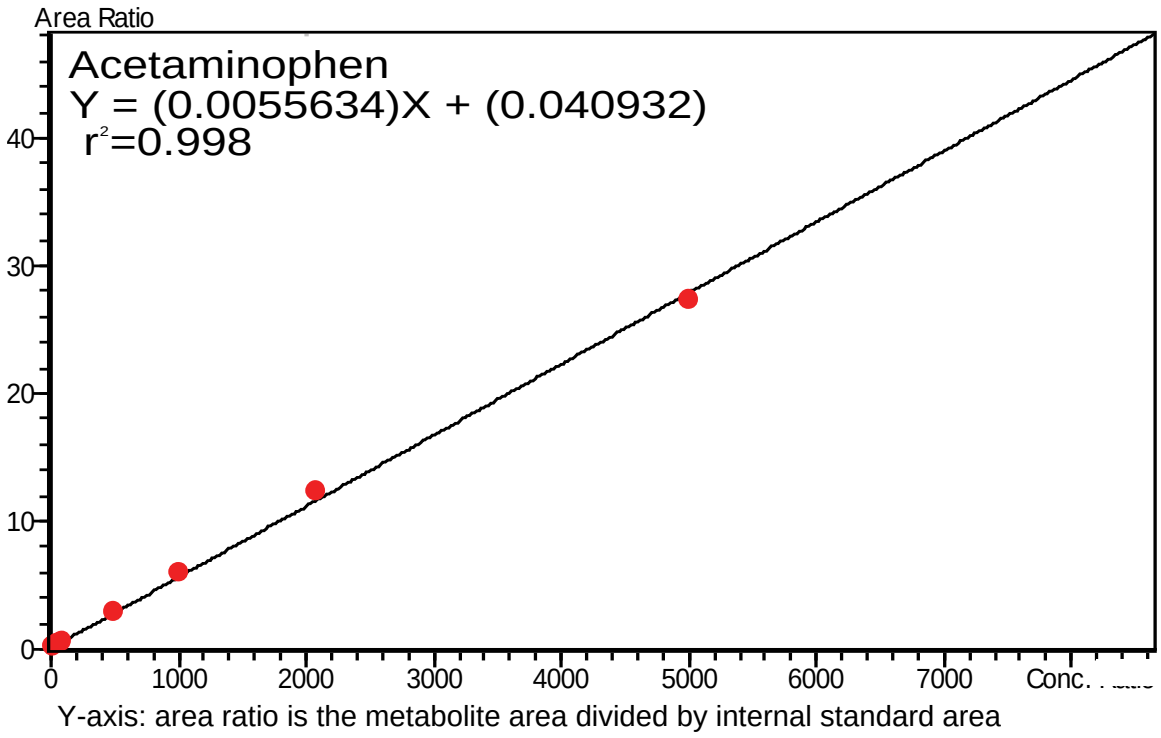
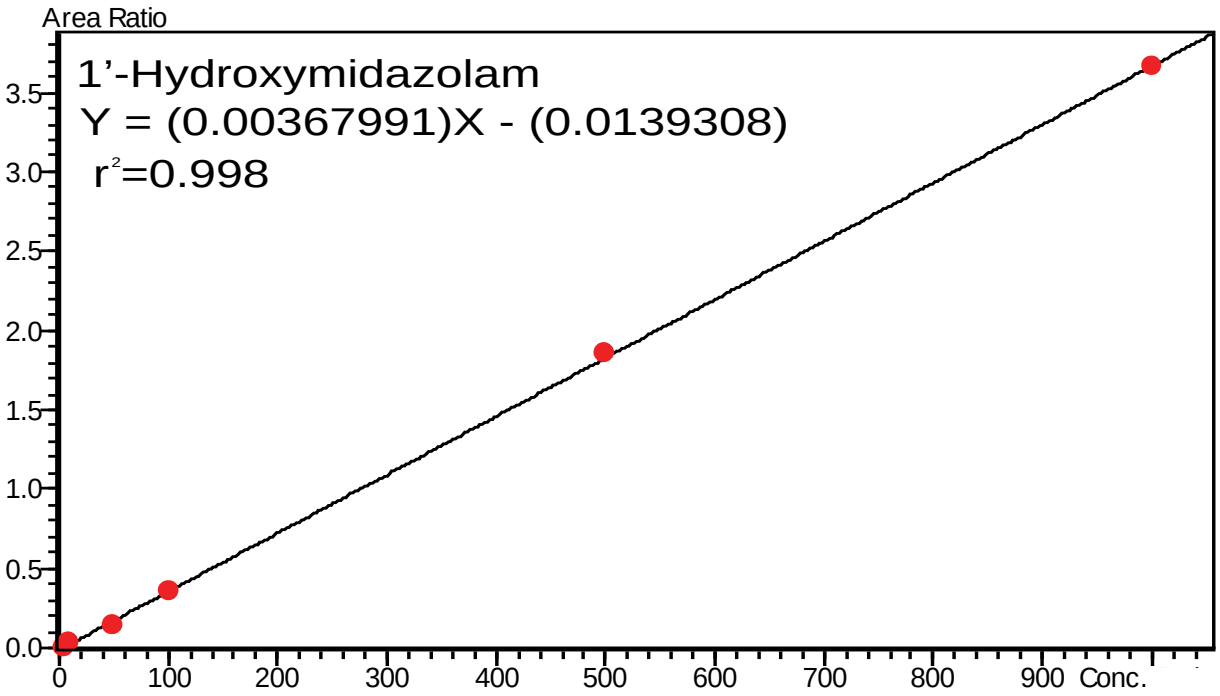


Figure 15. Standard curve for the UHPLC-MS-MS quantitative analysis of CYP1A2 substrate metabolite acetaminophen over the range of 0.1 to 100 ng/mL.



Y-axis: area ratio is the metabolite area divided by internal standard area

Figure 16. Standard curve for the UHPLC-MS-MS quantitative analysis of CYP3A4 substrate metabolite 1'-hydroxymidazolam over the range 0.01 to 10 ng/mL.

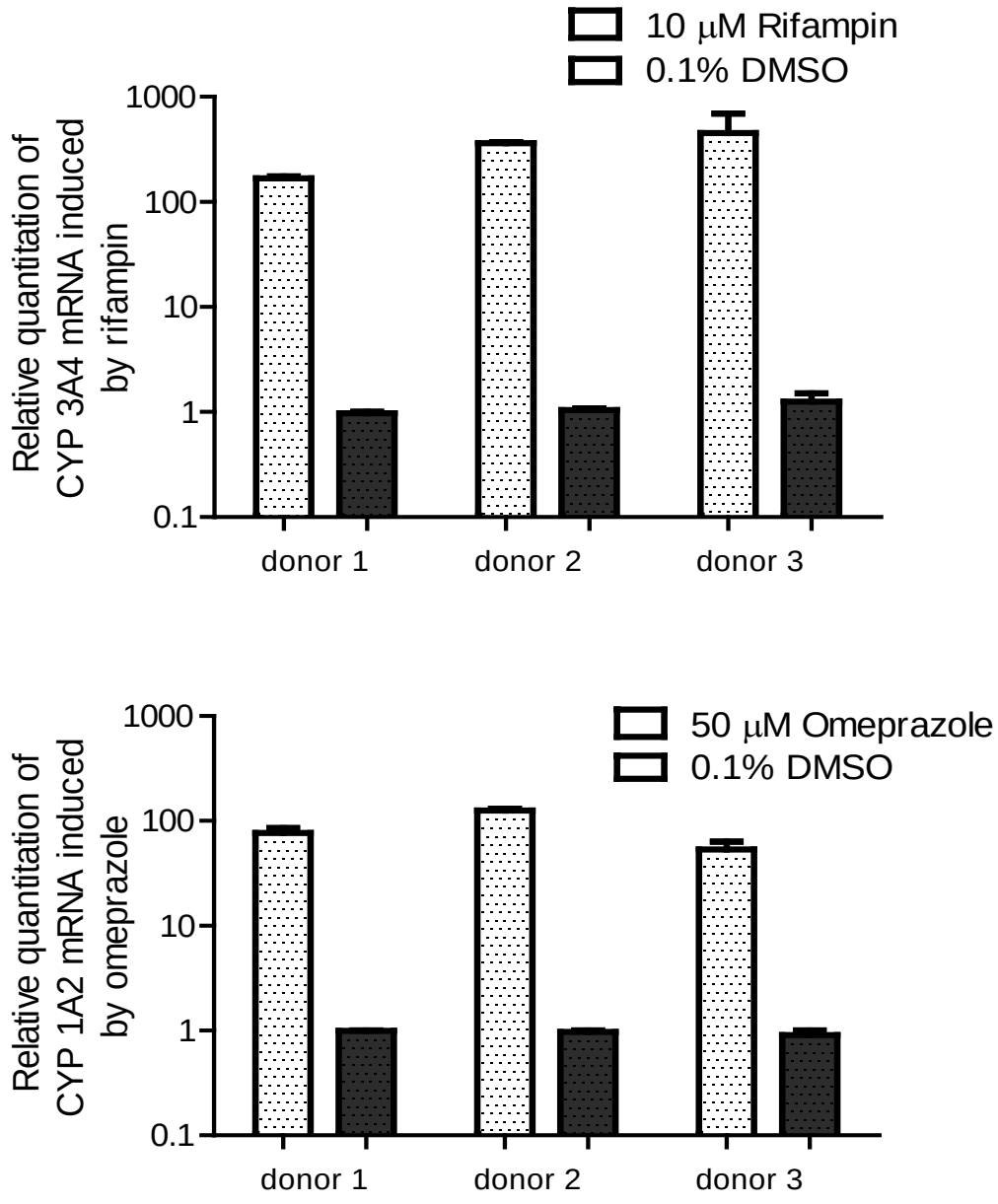


Figure 17. Basal inducible potential of mRNAs for CYP3A4 (top) and CYP1A2 (bottom) in hepatocytes from 3 different human donors. Induction of mRNA by standards is expressed relative to DMSO controls.

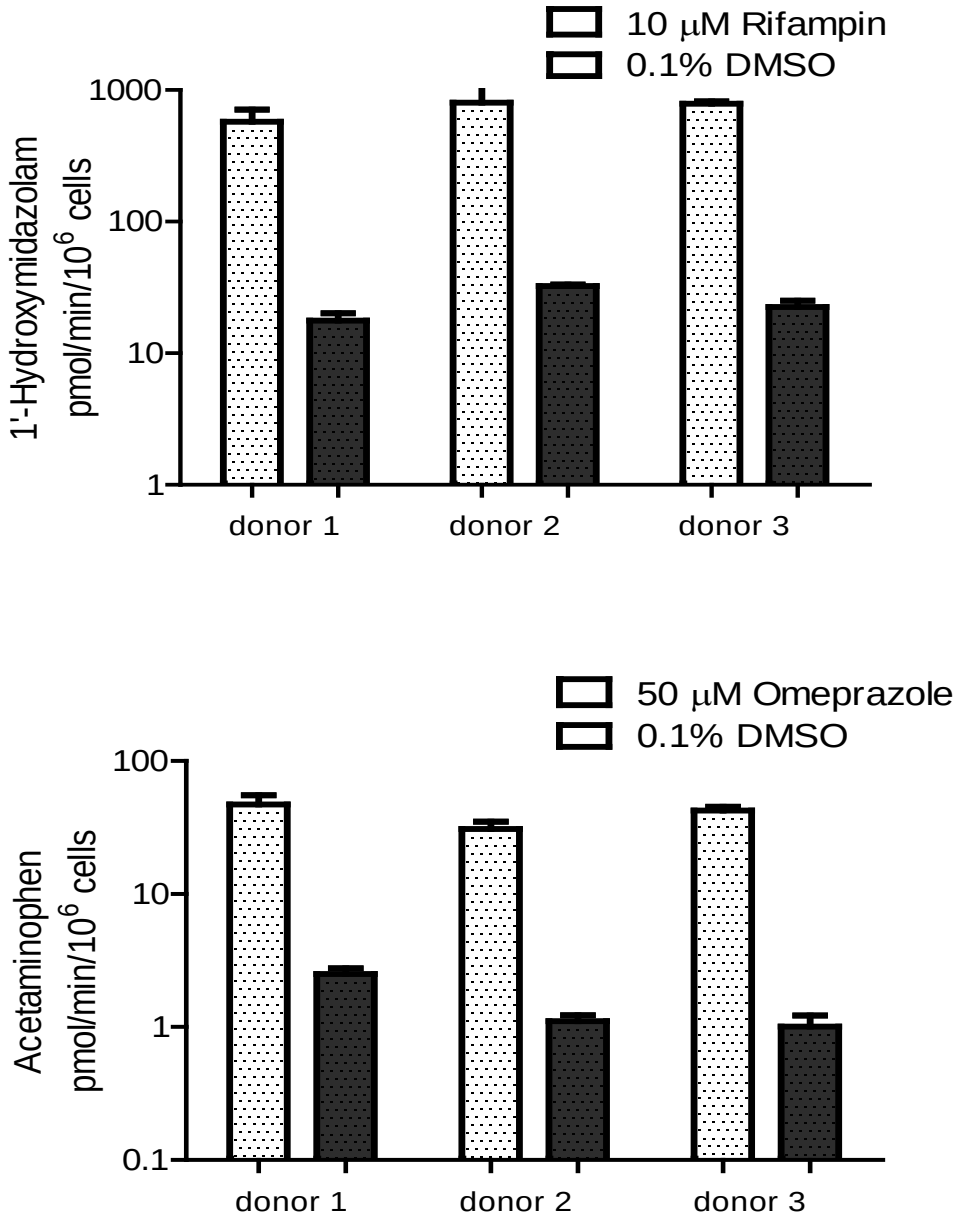


Figure 18. Basal inducible potential of CYP3A4 (top) and CYP1A2 (bottom) activities in hepatocytes from 3 different human donors. Enzyme activities were determined using UHPLC-MS-MS and are expressed relative to DMSO controls.

hop extract was ~33%.The concentration range selected represented the clinical relevant concentration but was also limited by the cytotoxicity of the hop extract to hepatocytes. As shown in Figure 19, the hop extract at low concentration (80 and 400 ng/mL) did not induce CYP1A2 and CYP3A4 mRNA. Also as shown in Figure 20 and Table VI, no induction of enzyme activity was observed in hepatocytes from any of the three donors compared with positive control.

When the hop extract concentration was increased to 2000 ng/mL, the CYP1A2 mRNA level showed a 2-fold increase while CYP 3A4 showed an 8-fold increase compared with the negative control (Figure 19). However, when compared with the omeprazole positive control (Figure 17), the increase in expression was negligible. The enzyme activity for CYP1A2 was induced 2.5-fold, which was almost the same as the increase in mRNA expression (Table VI). The CYP3A4 enzyme activity was increased ~2 fold (Table VI), which was similar but less than the 8-fold enhancement in mRNA level.

When the concentration of hop extract in the hepatocyte incubation was increased to 5000 ng/mL, CYP1A2 mRNA expression increase 5-fold and CYP3A4 expression increased 34-fold compared with the DMSO negative control (Figure 19). As an alternative measure of induction, the enzyme activity of CYP1A2 increased 3.5-fold and activity of CYP3A4 increased 2.9-fold (Table VI).

TABLE VI

FOLD INDUCTION OF CYP3A4 AND CYP1A2 ENZYME ACTIVITY IN HUMAN HEPATOCYTES BY A STANDARDIZED HOP EXTRACT COMPARED WITH NEGATIVE CONTROL (0.1% DMSO)

hops extract (ng/ml)	donor 1	donor 2	donor 3	donor 1	donor 2	donor 3
	CYP 3A4			CYP 1A2		
80	1.13	1.08	0.97	0.98	1.03	1.02
400	1.12	1.17	1.39	1.53	1.18	1.32
2000	1.59	1.98	2.07	2.94	1.86	2.49
5000	2.62	3.39	2.68	5.6	2.36	2.75

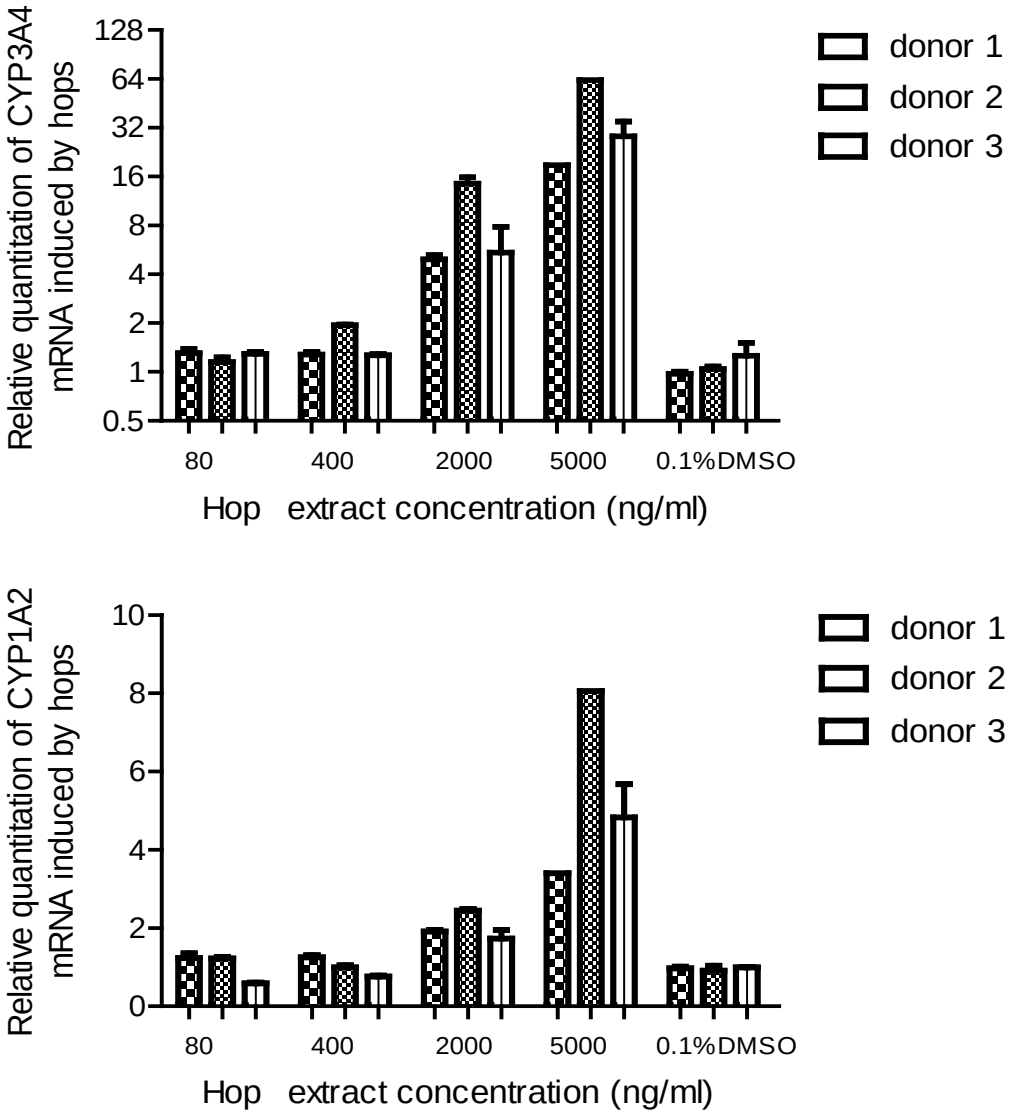


Figure 19. Induction of CYP3A4 (top) and CYP1A2 (bottom) mRNA from 3 human donors hepatocytes treated with hop extract relative to negative control (0.1% DMSO).

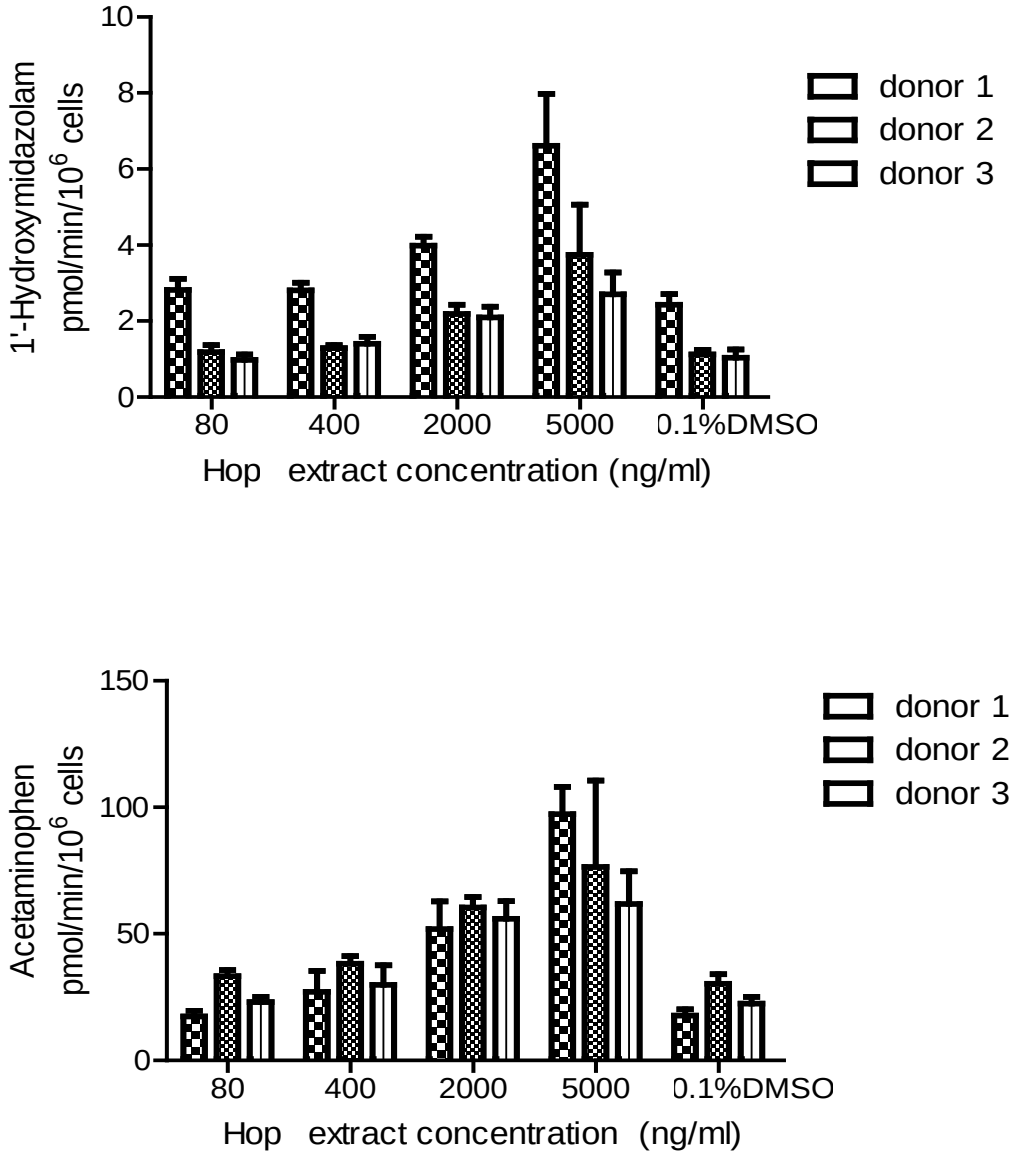


Figure 20. UHPLC-MS-MS quantitation of enzyme activity (metabolite formation) of CYP3A4 (top) and CYP1A2 (bottom) from 3 human donors hepatocytes treated with hop extract compared to negative control (0.1% DMSO).

According to FDA guidance (110), a drug that produces a change that is equal to or greater than 40% of the positive control (eq. (1)) may be considered to be an enzyme inducer *in vitro,* and *in vivo* evaluation would be warranted. Table VII summarizes the enzyme induction data for the hop treatment of human hepatocytes. None of the hop concentration induced CYP3A4 or CYP1A2 activities by more than 15% and most levels of hops enhanced enzyme activities by less than 10%.

$$\% \text{ positive control} = \frac{(\text{activity of test drug treated cells} - \text{activity of negative control}) \times 100}{(\text{activity of positive control} - \text{activity of negative control})} \quad \text{eq. (1)}$$

3.3.3 Cell viability

The cell viability study indicated no differences between the hepatocytes treated with hop extract up to 2000 ng/mL and the negative control (0.1% DMSO). There was a slight decrease in cell viability when hepatocytes were treated with the hop extract at 5000 ng/mL (Figure 21). However, there was still no significant difference between the hop-treated cells and the negative controls.

TABLE VII

PERCENTAGE INDUCTION OF CYP3A4 AND CYP1A2 ENZYME ACTIVITY IN HUMAN HEPATOCYTES BY A STANDARDIZED HOP EXTRACT COMPARED WITH POSITIVE CONTROL (KNOWN INDUCER)

hops extract (ng/ml)	donor 1	donor 2	donor 3	donor 1	donor 2	donor 3
	CYP 3A4			CYP 1A2		
80	0.95	0.31	0.03	-0.03	0.17	0.14
400	0.93	0.64	0.97	1.73	0.81	1.02
2000	3.56	3.69	2.63	6.21	3.69	4.45
5000	9.28	9.01	4.11	14.48	5.77	5.22

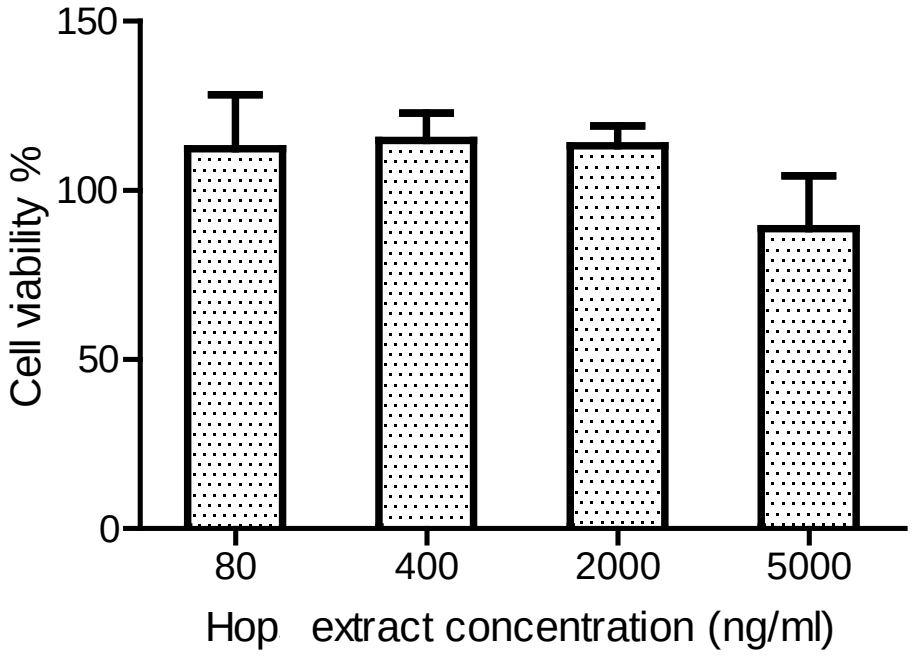


Figure 21. Cell viability measurement after 3 days treatment of various concentrations of a standardized hop extract.

3.4 Discussion

One of the most important drug-botanical interaction is the potential for a botanical to induce cytochrome P450 enzymes resulting in an adverse drug interaction (151). A number of xenobiotics, toxicants, and tobacco smoking can induce members of the CYP family (22), and parallel induction of the enzymes CYP1A1, 1A2, and 1B1, as well as other phase II metabolizing enzymes, is mediated through the aryl hydrocarbon receptor (AHR). Induction of CYP3A4, along with several other Phase I and Phase II drug-metabolizing enzymes, is pregnane X receptor (PXR) mediated.

Fresh or cryopreserved human hepatocytes are used as standard models for predicting human P450 enzyme induction *in vitro* and also highly recommended by the FDA for this purpose. Cryopreserved hepatocytes were used during this dissertation because they have some advantages over freshly isolated cells. For example, individual lots can be evaluated for induction using known inducers, and high-quality inducible lots of cells can be stored and used for evaluation of test articles. Also, cryopreserved hepatocytes are convenient, more easily available, and able to provide multiple lots for comparison.

There are several methods for evaluating of CYP induction. Besides in situ enzyme activity measurement by LC-MS-MS and mRNA levels measurement using RT-PCR, western blots are used for relative quantitative determination of specific P450 proteins. Also receptor gene assays for receptors mediating induction of P450 enzymes are also widely used. There is a possibility that the test article may function both as inducer and inhibitor, and the co-occurrence of cytochrome P450 inhibition and induction can confound induction results. The inhibition of CYPs can minimize induction effects when using enzyme activity as the only

end point. When evaluating drug metabolizing enzyme induction as a possible drug-botanical interactions, mRNA expression determination would help to distinguish between induction of gene expression and protein stabilization. In this dissertation, a small increase in CYP3A4 and CYP1A2 enzyme activity treated with the hop extract was observed. However, this response was not significant compared with the positive control. Measurement of mRNA levels provided similar results and confirmed the enzyme activity data. It should also be noted that cytotoxicity of the test agent can decrease cell number and artifactually lower mRNA levels. Thus, cytotoxicity should also be evaluated. In the present study, a cell proliferation assay was used to determine the viable cells after treatment with the hop extract. The data suggested only minor toxicity even at the highest concentration used. Therefore, the lack of observable cytochrome P450 enzyme induction by hops was not caused by loss of viable cells during the assays.

3.4 Conclusions

The cytochrome P450 induction assays carried out in human hepatocytes during this dissertation were validated using known inducers. Assays carried out using human hepatocytes from 3 different donors showed greater than 28-fold induction of CYP1A2 and 23-fold induction of CYP3A4. The mRNA levels were consistent with enzyme activities. When these assays were applied to the investigation of induction of CYP1A2 and CYP3A4 by various concentration of a hop extract, no significant cytochrome P450 enzyme induction was observed.

CHAPTER 4

INHIBITION STUDIES OF PRENYLFLAVONOIDS FROM HOPS EXTRACT ON HUMAN CYTOCHROME P450s

4.1 Introduction

The female flowers of hops (*Humulus lupulus* L.) are used in the brewing industry to add aroma and bitterness to beer. Recently, there has been interest in the possible health benefits of hops. Hops has been used as a mild sedative, but most of the recent research has focused on its potential estrogenic and chemopreventive properties. Among the possible active constituents, prenylated flavonoids have received the most attention. Chemically, they can be divided into two groups: prenylated chalcones and prenylated flavanones. In hop cones, the most abundant prenylated chalcone is xanthohumol (XN; Figure 6) which can be up to 1% of dry weight (78, 152). XN has been studied primarily for its chemopreventive properties. It exhibits strong anti-proliferative activity against breast, colon and ovarian cancer cell lines and is a potent inducer of quinone reductase (80, 81, 153). Among prenylated flavanones, 8-prenylnaringenin (Figure 6) has been identified as one of the most potent phytoestrogens (102), and its estrogenic properties have been confirmed in numerous *in vitro* and animal studies (141, 154, 155). Isoxanthohumol (IX), the 5-*O*-methyl derivative of 8-PN, has much weaker estrogenic activity (154). However, several *in vitro* and *in vivo* studies have shown that IX can be metabolically converted into 8-PN either by the action of cytochrome P450 (CYP) enzymes or by intestinal micro biota (142, 144, 156, 157). Thus, IX can be considered a

pro-estrogen, which provides rationale for inclusion of this compound in the standardization of hop extracts.

Because women are using hop-based supplements (76), it is important to understand the potential of these supplements to interact with clinically used drugs. Besides induction of the drug metabolizing cytochrome P450 enzymes described in Chapter 3, inhibition of these enzymes is another important type of drug-botanical interaction. Inhibition of CYP enzymes by hop prenylflavonoids has been reported by Henderson *et al.* who focused on the P450s involved in carcinogen activation, especially CYP1A1, CYP1B1, and CYP1A2 (88). Similarly, Hemachandra *et al*. reported that estrogen-induced expression of CYP1B1 and CYP1A1 was attenuated by a hop extract and that 8PN was a potent inhibitor of CYP1A1 and CYP1B1 (158). Hop-containing beers have been examined for their ability to affect CYP enzymes and P-glycoprotein. Most products showed some inhibition of CYP2C9 (159), however, individual compounds responsible for this inhibition were not identified. These findings suggest that studies of possible of inhibition of drug-metabolizing CYP enzymes by hops and hop constituents are needed. The results of such additional investigations would be useful for the prediction of *in vivo* drug-botanical interactions and the design of follow-up clinical studies.

In this dissertation, a rapid, selective, and sensitive method using UHPLC-MS-MS was developed to investigate *in vitro* human cytochrome P450 inhibition by an ethanolic extract of hops as well as by 4 prenylated flavonoids from hops using pooled human liver microsomes as the model system.

4.2 Materials and methods

4.2.1 Chemicals and reagents

All organic solvents were HPLC grade or better and were purchased from Thermo Fisher (Hanover Park, IL). Purified water was prepared by using a Millipore Milli-Q purification system (Millipore, Billerica, MA). All substrates phenacetin, bupropion, amodiaquine, tolbutamide, S-(+)-mephenytoin, dextromethorphan, chlorzoxazone, midazolam, and their metabolites acetaminophen, OH-bupropion, desethylamodiaquine, OH-tolbutamide, 4-OH-mephenytoin, dextrorphan, 6-OH-chlorzoxazone, 1'-OH-midazolam, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), magnesium chloride ($MgCl_2$), ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St.Louis, MO). Stable isotope labeled internal standards acetaminophen-d_4, OH-bupropion-d_6, desethylamodiaquine-d_3, OH-tolbutamide-d_9, 4-OH-mephenytoin-d_3, dextrorphan-d_3, 6-OH-chlorzoxazone-d_2, flurazepam were purchased from Cerilliant (Round Rock, TX) and BD Gentest (Woburn, MA). Human liver microsomes (HLMs) (50 donors mixed gender pool; protein concentration 20 mg/mL; CYP total activity 340 pmol/(min x mg)) were purchased from BD Biosciences (San Jose, CA). The hop extract examined in this study was the same as that used in a recent Phase I clinical trial carried out in the UIC/NIH Center for Botanical Dietary Supplements Research, University of Illinois at Chicago. The extract was standardized by using LC-MS-MS analysis to contain 33.84% XN, 0.35% 8-PN, 1.77% 6-PN, and 1.07% IX. Details on the preparation of this extract was not part of this dissertation and will be reported elsewhere. XN was isolated from hops and purified as previously described (140). The purity was determined to be >99.5% by qHNMR. IX (> 99% pure by qHNMR) was prepared by cyclization of XN as

described previously (152). 8-PN was chemically synthesized, and 6-PN was purified as previously reported (160).

4.2.2 Screening for inhibitors of cytochrome P450 isozymes

Incubation mixtures (100 µl) contained 0.1 mg/mL HLMs, 1 mM NADPH, various amounts of the inhibitor being tested, and a marker substrates (see concentrations in Table VIII) in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 5 mM $MgCl_2$ and 1 mM EDTA. All test articles were dissolved in methanol (final concentration < 1%). After pre-incubation at 37°C for 5 min, the reactions were initiated by addition of 1 mM NADPH. Cytochrome P450 substrates and incubation conditions with HLMs areare summarized in Table VIII. Enzymatic reactions were carried out under conditions shown to be linear with respect to time, HLM protein concentration, and substrate concentration (at the apparent Km concentration). After incubation, 400 µl acetonitrile containing internal standards was added to stop the reaction. The samples were vortexed for 2 min and centrifuged at 13,000 x *g* at 4°C for 10 min. After centrifugation, 450 µl supernatant was transferred to a new micro-centrifuge tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 µl 20% aqueous acetonitrile, and 5 µl was injected onto the column for analysis using UHPLC-MS-MS.

4.2.3 IC_{50} determination

When cytochrome P450 enzyme inhibition by a compound exceeded 50% at 10 µM, follow-up experiments were carried out to determine the IC_{50} value for each active botanical

compound. The IC_{50} value was determined by measuring the CYP isoform activity at 8 inhibitor concentrations spanning 4 orders of magnitude. The marker substrate concentrations were set at their respective Km values. HLM incubations were carried out using individual substrates as described above. Comparison was made with negative control incubations containing no inhibitor, and activity was expressed as the percentage of control activity remaining.

4.2.4 UHPLC-MS-MS

Formation of metabolites from probe substrates was measured using UHPLC-MS-MS on a Shimadzu (Kyoto, Japan) Nexera UHPLC system interfaced with a Shimadzu LCMS-8030 triple quadrupole mass spectrometer. Analytes were separated on a Shimadzu Shim-pack XR-ODS III UHPLC column (2.0 x 50 mm, 1.6 µm) using a 2 min linear gradient from 5-100% acetonitrile in 0.1% aqueous formic acid. The UHPLC column was re-equilibrated at 5% acetonitrile for 1 min before the next injection. The total run time including equilibration was 3 min. The flow rate was 0.5 mL/min. Since some metabolites required positive ion electrospray and others required negative ion electrospray, polarity switching was used so that all species could be measured during a single UHPLC-MS-MS analysis. The SRM transitions that were used are summarized in Table IX. The dwell time was 20 ms/ion and the polarity switching time was 15 ms. The mass spectrometer source parameters were as follows: DL temperature 300°C, the spray voltage 3500 V, the nebulizing gas flow 3 L/min, and the drying gas flow 20 L/min.

TABLE VIII

CYTOCHROME P450 SUBSTRATES AND INCUBATION CONDITIONS WITH HUMAN LIVER MICROSOMES (0.1 MG/ML) FOR EACH SPECIFIC ISOFORM

CYP	Substrate	Concentration (μM)	Incubation Time
1A2	Phenacetin	80	15
2B6	Bupropion	20	15
2C8	Amodiaquine	2	15
2C9	Tolbutamide	100	12
2C19	S-(+)-Mephenytoin	30	30
2D6	Dextromethorphan	3	12
2E1	Chlorzoxazone	40	15
3A4	Midazolam	2	5

TABLE IX

SELECTION OF PRECURSOR AND PRODUCT IONS OF PROBE SUBSTRATES' MAKER METABOLITES AND INTERNAL STANDARDS

CYP isoform	Compound	SRM (*m/z*)	Electrospray polarity	Q1	CE	Q3
		Metabolite				
1A2	Acetaminophen	152.0>110.0	+	-17	-20	-20
2B6	OH-Bupropion	256.0>238.0	+	-19	-10	-26
2C8	Desethylamodiaquine	328.0>283.0	+	-17	-15	-19
2C9	OH-Tolbutamide	285.0>186.1	-	20	20	21
2C19	4-OH-Mephenytoin	235.0>149.9	+	-12	-20	-14
2D6	Dexthorphan	258.1>133.1	+	-19	-45	-18
2E1	6-OH-Chlorzoxazone	183.9>120.1	-	19	20	22
3A4	1'-OH-Midzaolam	342.0>203.2	+	-17	-25	-21
		Internal standard				
1A2	Acetaminophen-d_4	156.0>114.0	+	-17	-15	-20
2B6	OH-Bupropion-d_6	262.1>244.3	+	-19	-10	-26
2C8	Desethylamodiaquine-d_3	333.3>283.3	+	-17	-20	-19
2C9	OH-Tolbutamide-d_9	294.1>186.1	-	14	20	22
2C19	4-OH-Mephenytoin-d_3	238.0>150.0	+	-12	-25	-17
2D6	Dextrorphan-d_3	261.1>133.1	+	-13	-45	-24
2E1	6-OH-Chlorzoxazone-d_2	185.9>122.1	-	20	20	24
3A4	Flurazepam	388.1>315.1	+	-20	-30	-30

4.2.5 Statistical analysis

All of the experiments were carried out in triplicate, and all data are expressed as the mean ± SD. The statistical analysis of these results consisted of a student t-test and ANOVA using GraphPad Prism 5.0. Peak areas were determined and evaluated using Shimadzu quantitation software. Inhibition curves of percent control activity versus the logarithm of the test compound concentrations were constructed, and IC_{50} values were calculated via exponential decay with single, four-parameter curve fitting analysis using Sigma Plot 8.0 software (Systat Software, Chicago, IL).

4.3 Results

4.3.1 UHPLC-MS-MS

Separation of probe substrates, their metabolites, and the corresponding internal standards are shown in Figures 23 to 26. The probe substrates for each cytochrome P450 enzyme were selected because they are recommended by the FDA guidance for industry (110) and exhibit robust turnover, high selectivity for one CYP enzyme, and good solubility. Figure 22 shows the positive ion electrospray UHPLC-MS-MS analysis of acetaminophen and OH-bupropion, which are CYP1A2 and CYP2B6 metabolites of the probe substrates phenacetin and bupropion, respectively. Note that the separation of these and all other metabolites was completed within 2 min. Figure 23 shows a representative UHPLC-MS-MS analysis of desethylamodiaquine and OH-tolbutamide, which are CYP2C8 and CYP2C9 metabolites of the respective probe substrates amodiaquine and tolbutamide. Figure 24 shows the separation of 4-OH-mephenytoin and dexthorphan, which are CYP2C19 and CYP2D6

metabolites of the their probe substrates mephenytoin and dextromethorphan. Finally, Figure 25 shows the analysis of metabolites 6-OH-chlorzoxazone and 1'-OH-midzaolam, which were formed from chlorzoxazone and midazolam by CYP2E1 and CYP3A4, respectively. Note that stable isotope labeled internal standards of each metabolite were added to each sample after incubation with HLMs to control for sample losses during handling, instrument variations between analyses, and to compensate for possible matrix effects that might cause ion suppression or enhancement. These surrogate standards coeluted or nearly coeluted with their respective metabolites during UHPLC-MS-MS and are also shown in Figures 22 to 25.

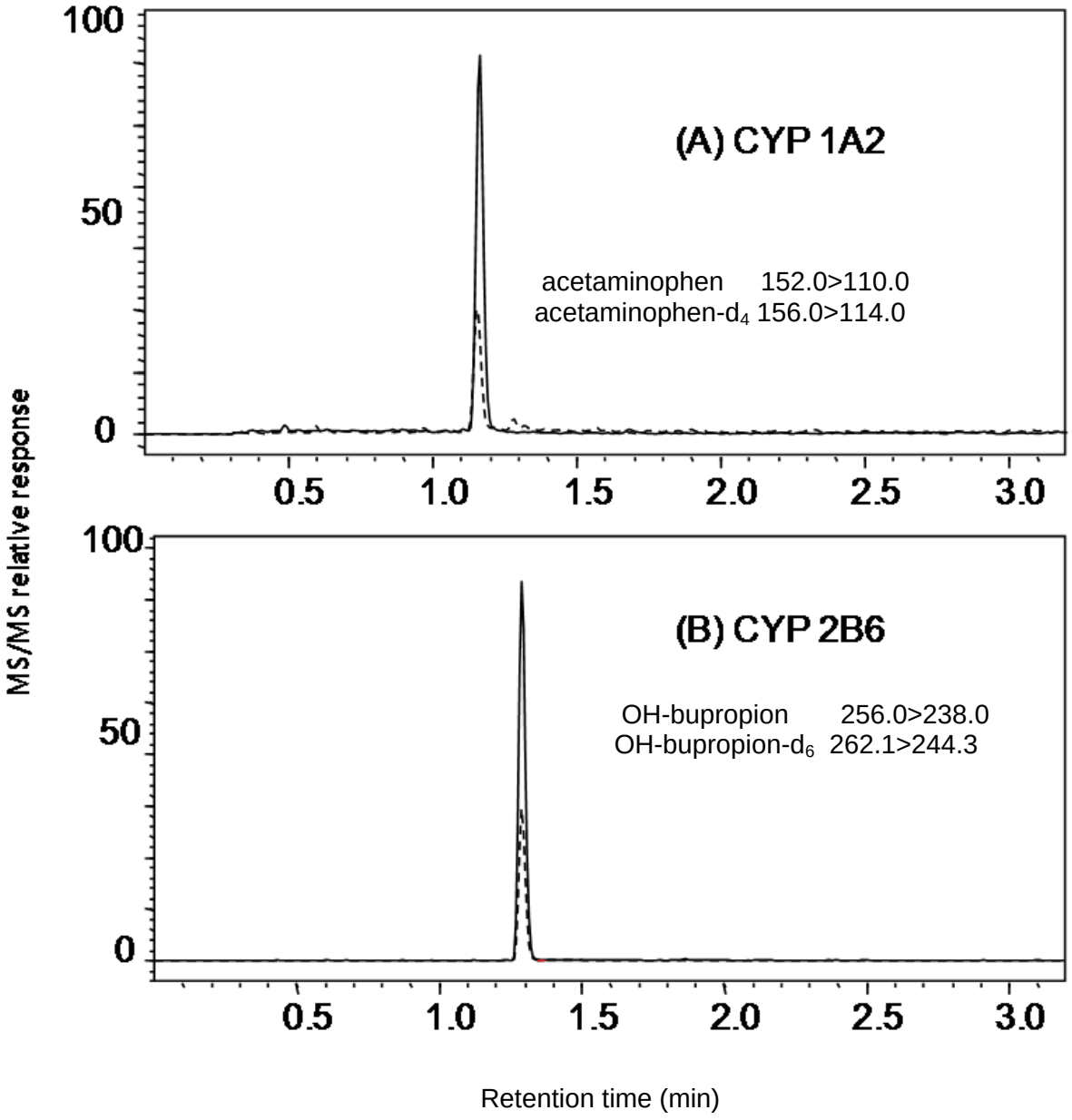


Figure 22. Positive ion electrospray UHPLC-MS-MS SRM chromatograms of acetaminophen and OH-bupropion, which are CYP1A2 and CYP2B6 metabolites of the probe substrates phenacetin and bupropion. The dashed lines indicate coelution of the stable isotope labeled surrogate standards. (A) CYP1A2 conversion of phenacetin to acetaminophen; and (B) CYP2B6 conversion of bupropion to OH-bupropion.

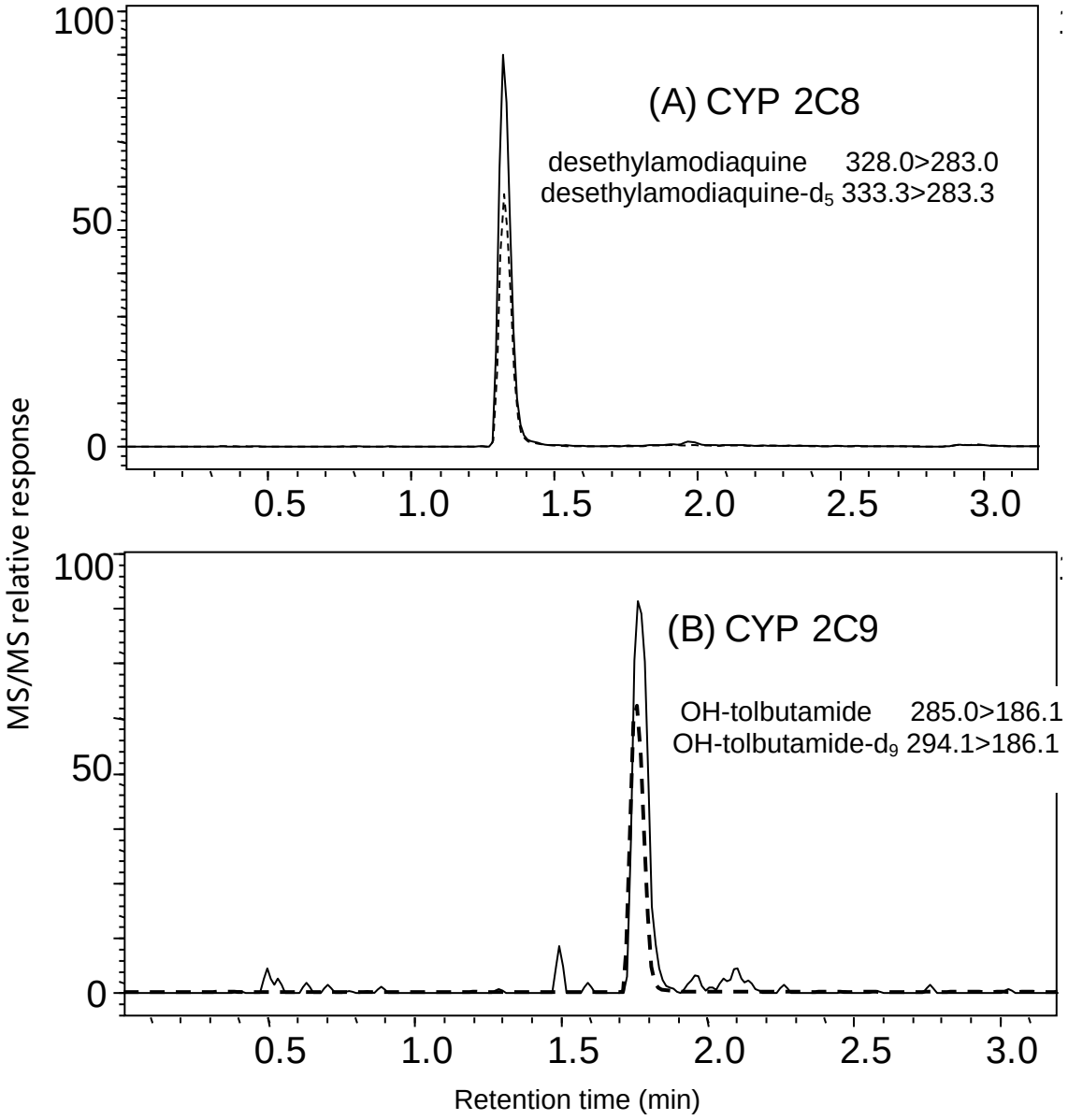


Figure 23. UHPLC-MS-MS SRM chromatograms of desethylamodiaquine and OH-tolbutamide which are CYP2C8 and CYP2C9 metabolites of the probe substrates amodiaquine and tolbutamide. Note that polarity switching was used during these measurements such that (A) the CYP2C8 metabolite desethylamodiaquine was measured using positive ion electrospray; and (B) the CYP2C9 metabolite OH-tolbutamide was measured using negative ion electrospray.

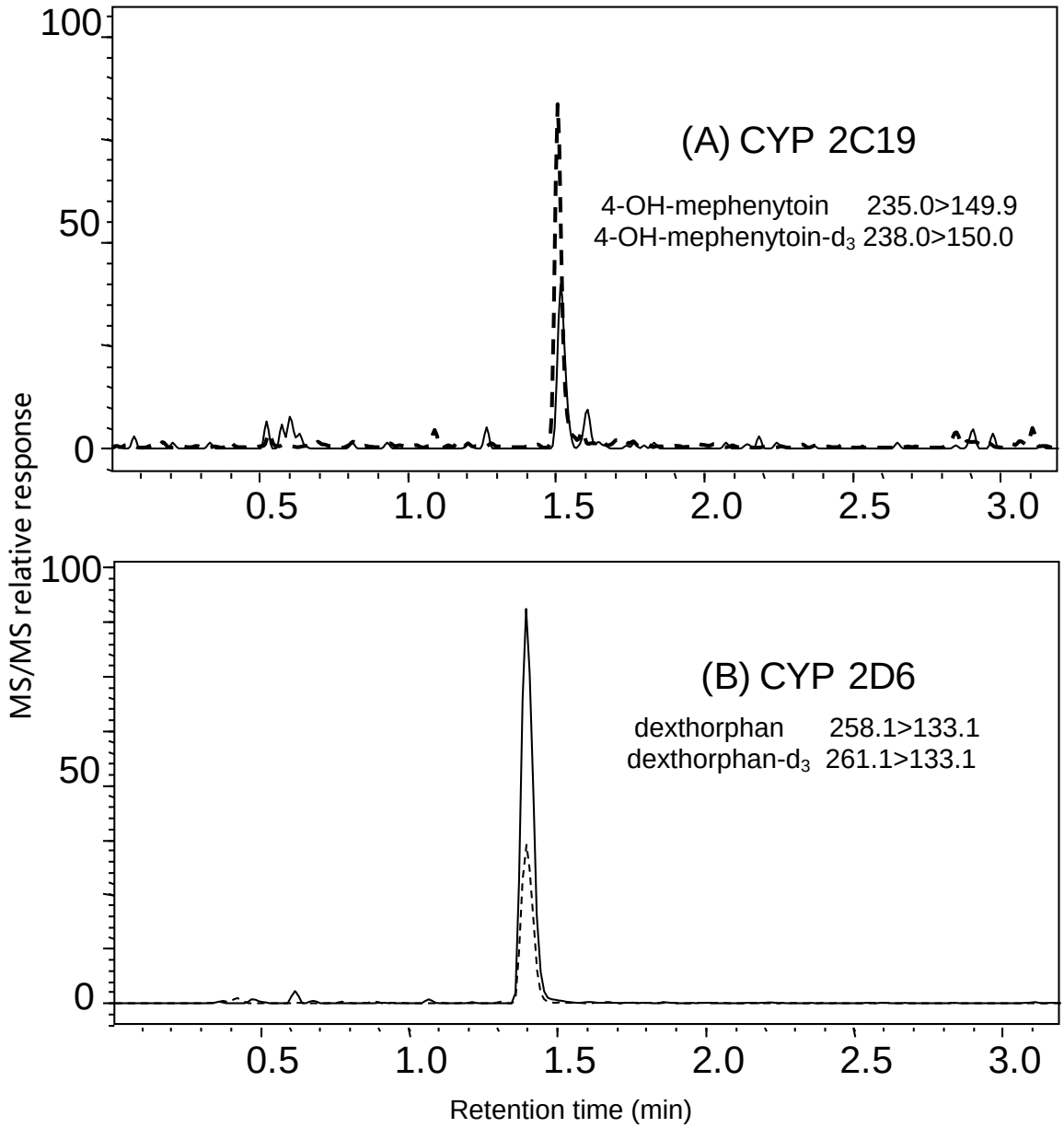


Figure 24. Positive ion electrospray UHPLC-MS-MS SRM chromatograms of 4-OH-mephenytoin and dexthorphan, which are CYP2C19 and CYP2D6 metabolites of the probe substrates mepheyntoin and dextromethorphan. The dashed lines indicate coelution of the stable isotope labeled surrogate standards. (A) CYP2C19 conversion of mephenytoin to 4-OH-mephenytoin; and (B) CYP2D6 conversion of dextromethorphan to dexthorphan.

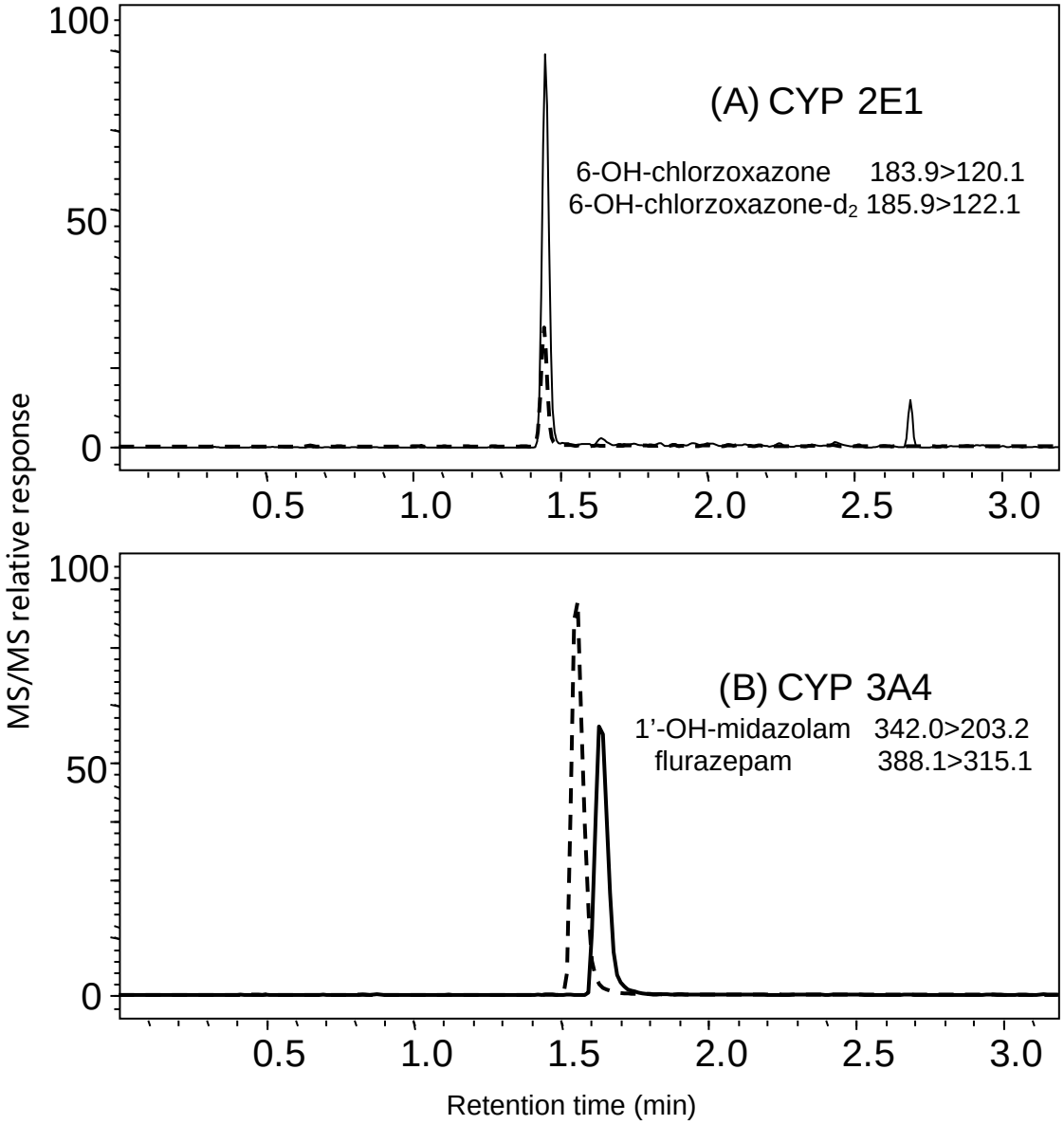


Figure 25. UHPLC-MS-MS SRM chromatograms of 6-OH-chlorzoxazone and 1'-OH-midazolam which are CYP2E1 and CYP3A4 metabolites of the probe substrates chlorzoxazone and midazolam. Note that polarity switching was used during these measurements such that (A) the CYP2E1 metabolite 6-OH-chlorzoxazone was measured using negative ion electrospray; and (B) the CYP3A4 metabolite 1'-OH-midazolam was measured using positive ion electrospray.

4.3.2 Initial screening of 8 CYP isoforms

During initial experiments, a hop extract and single prenylated flavonoids were tested for their ability to inhibit 8 major cytochrome P450 enzymes, which are responsible for the metabolism of the vast majority of clinically useful drugs. As summarized in Table X, 4 major prenylated flavonoids present in hops (XN, IX, 6-PN, and 8-PN) were tested individually at 1 μM and 10 μM for inhibition of CYP enzymes. At 10 μM, all 4 prenylated flavonoids showed >60% inhibition of CYP2C8 and CYP2C9. However, only IX and 8-PN were strong inhibitors (>90%) of CYP2C19 (Table X). 8-PN was also a strong (>88%) inhibitor of CYP1A2.

The hop extract at 5 μg/mL showed strong (≥70%) inhibition of CYP2C8, CYP2C9 and CYP2C19 but only weak inhibition of CYP2B6 (36%) and CYP1A2 (27%). Inhibition of the CYP2C family was probably due to the actions of multiple prenylated flavonoids which were present at high levels in the hop extract. The weak inhibition of CYP1A2 was probably the result of 8-PN, which is a potent inhibitor of CYP1A2 but present at only 0.35% of the hop extract. The inhibition of CYP2B6 by the hop extract might be produced mostly by as yet unknown constituents in the hop extract, since a mixture of all 4 tested prenylated flavonoids, whose concentrations in the mixture are the same as in the standardized hop extract which are 33.84% XN, 0.35% 8-PN, 1.77% 6-PN, and 1.07% IX, (of which 6PN, 8PN and XN were weak inhibitors) did not show any significant inhibition.

4.3.3 IC_{50} results

After these initial screening experiments, IC_{50} values were determined for the hop extract and individual hop prenylated flavonoids showing > 50% inhibition at 10 μM. Figure 26 shows

the IC50 curves for inhibition of CYP1A2, CYP2C8, CYP2C9, and CYP2C19 by the hop extract. Figure 27 shows the IC_{50} curves for the inhibition of pure XN and 6-PN of CYP2C8 and CYP2C9 , Figure 28 shows the IC_{50} curves for 8-PN inhibition of CYP1A2, CYP2C8, CYP2C9, and CYP2C19, and Figure 29 shows the IC_{50} curves for the inhibition CYP2C8, CYP2C9 and CYP2C19 by IX. The IC_{50} values were found to be as low as 0.17 ± 0.01 μM for IX inhibition of CYP2C8. The overall results are summarized in Table XI.

TABLE X

SCREENING OF PRENYLATED FLAVONOIDS FROM HOPS (1 µM AND 10 µM) AND A HOP EXTRACT (5 µG/ML) FOR INHIBITION OF CYTOCHROME P450 ENZYMES

	CYP1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4
				% Inhibition (1 µM)				
6-PN	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
8-PN	55.8 ± 0.7[a]	< 10	32.2 ± 1.2	ND	43.8 ± 0.2	< 10	< 10	< 10
IX	< 10	< 10	87.7 ± 1.1	31.6 ± 8.0	51.0 ± 8.6	< 10	< 10	< 10
XN	< 10	< 10	47.8 ± 5.1	< 10	< 10	< 10	< 10	< 10
				% Inhibition (10 µM)				
6-PN	21.4 ± 0.8	35.7 ± 1.9	85.5 ± 1.2	63.8 ± 2.0	14.3 ± 0.1	< 10	< 10	< 10
8-PN	88.4 ± 1.6	34.7 ± 1.7	97.6 ± 0.1	93.0 ± 0.6	92.8 ± 5.5	< 10	< 10	31.4 ± 2.8
IX	48.2 ± 4.6	< 10	98.9 ± 0.1	82.1 ± 1.7	96.3 ± 1.5	< 10	< 10	< 10
XN	11.8 ± 1.1	43.9 ± 1.3	92.9 ± 0.4	69.7 ± 3.0	15.0 ± 1.9	< 10	< 10	< 10
Hops[b]	26.7 ± 8.1	36.4 ± 5.1	92.7 ± 0.4	88.1 ± 2.5	69.8 ±10.8	19.5 ± 2.5	13.7 ± 12.6	19.2 ± 2.9
4 Mix[c]	16.4 ± 0.7	14.9 ± 1.4	92.7 ± 0.6	75.9 ± 2.1	65.5 ± 6.8	20.8 ± 4.9	< 10	29.2 ± 2.3

[a] Data expressed as mean ± SD

[b] Hop extract tested at 5 µg/mL

[c] Mixture of 6-PN, 8-PN, IX, and XN at concentrations the same as in standardized hop extract

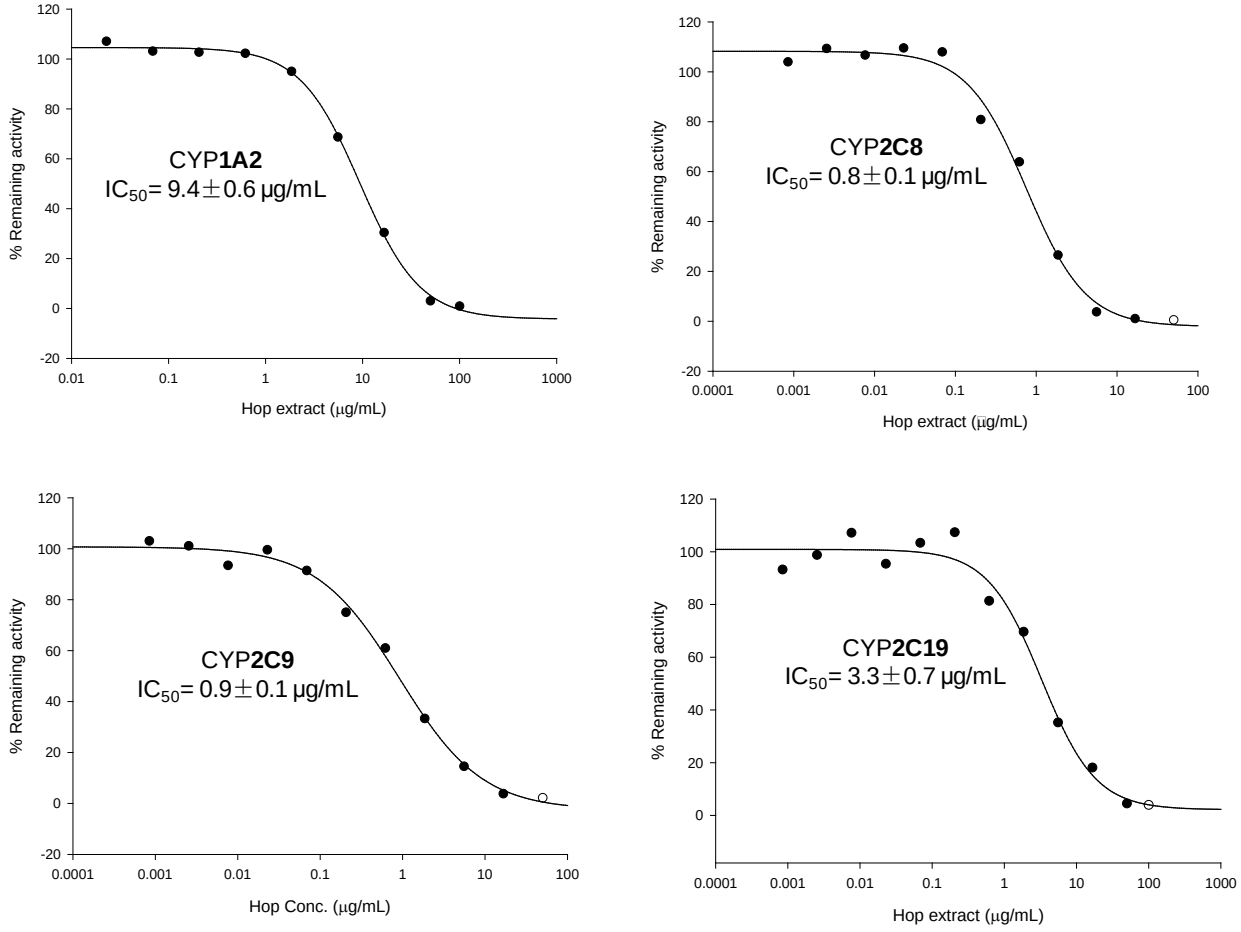


Figure 26. IC50 curves showing inhibition by the hop extract of CYP1A2, CYP2C8, CYP2C9, and CYP2C19.

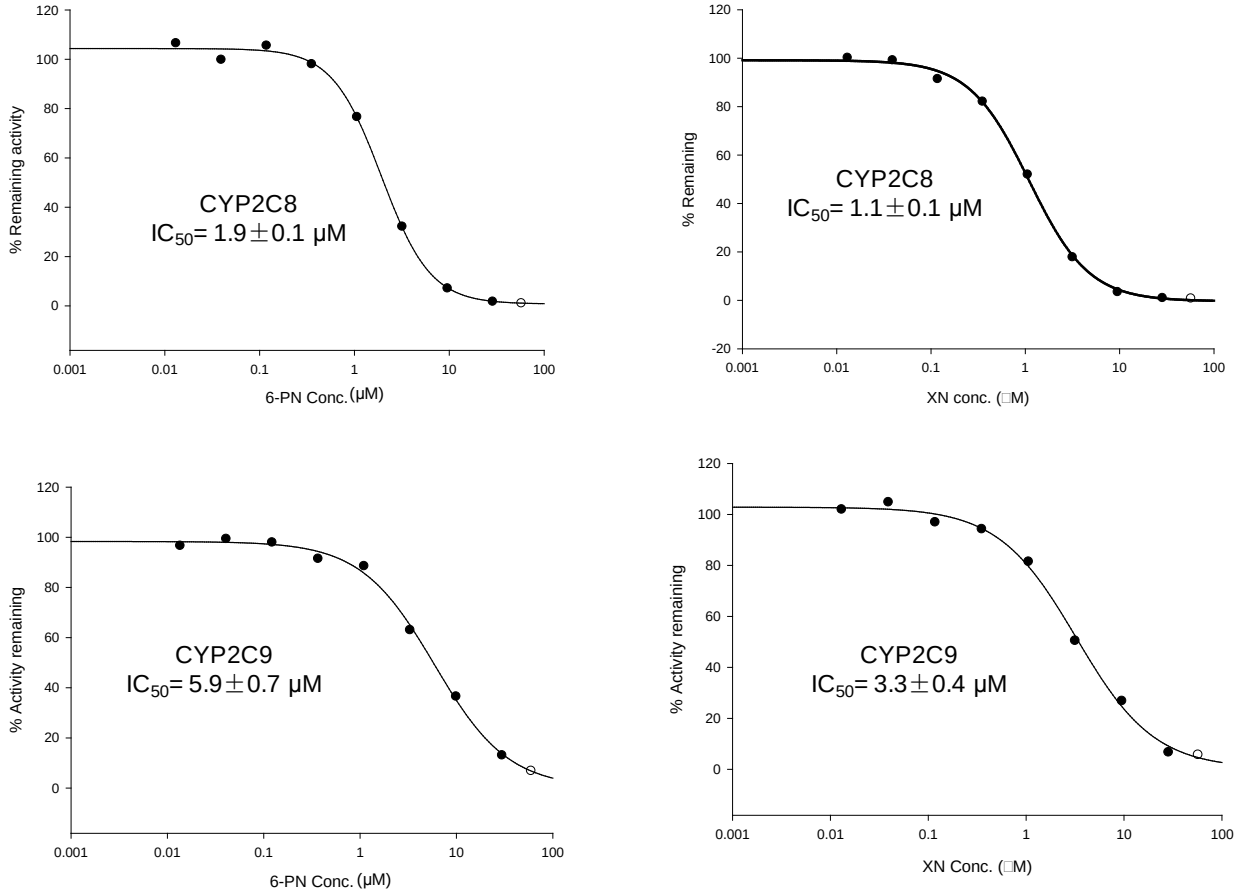


Figure 27. IC_{50} curves for inhibition of CYP2C8 and CYP2C9 by 6-PN and XN.

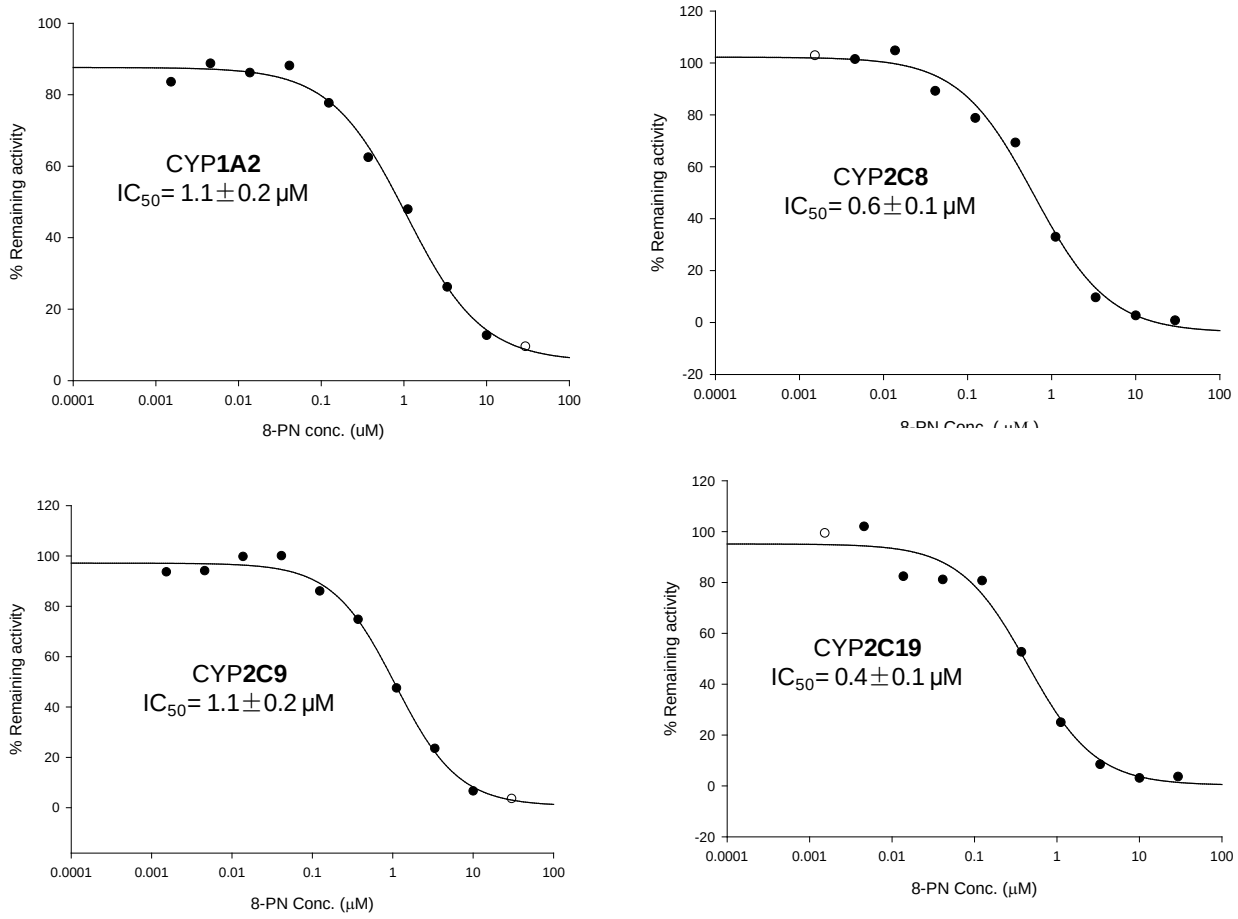


Figure 28. IC_{50} curves for 8-PN inhibition of CYP1A2, CYP2C8, CYP2C9, and CYP2C19.

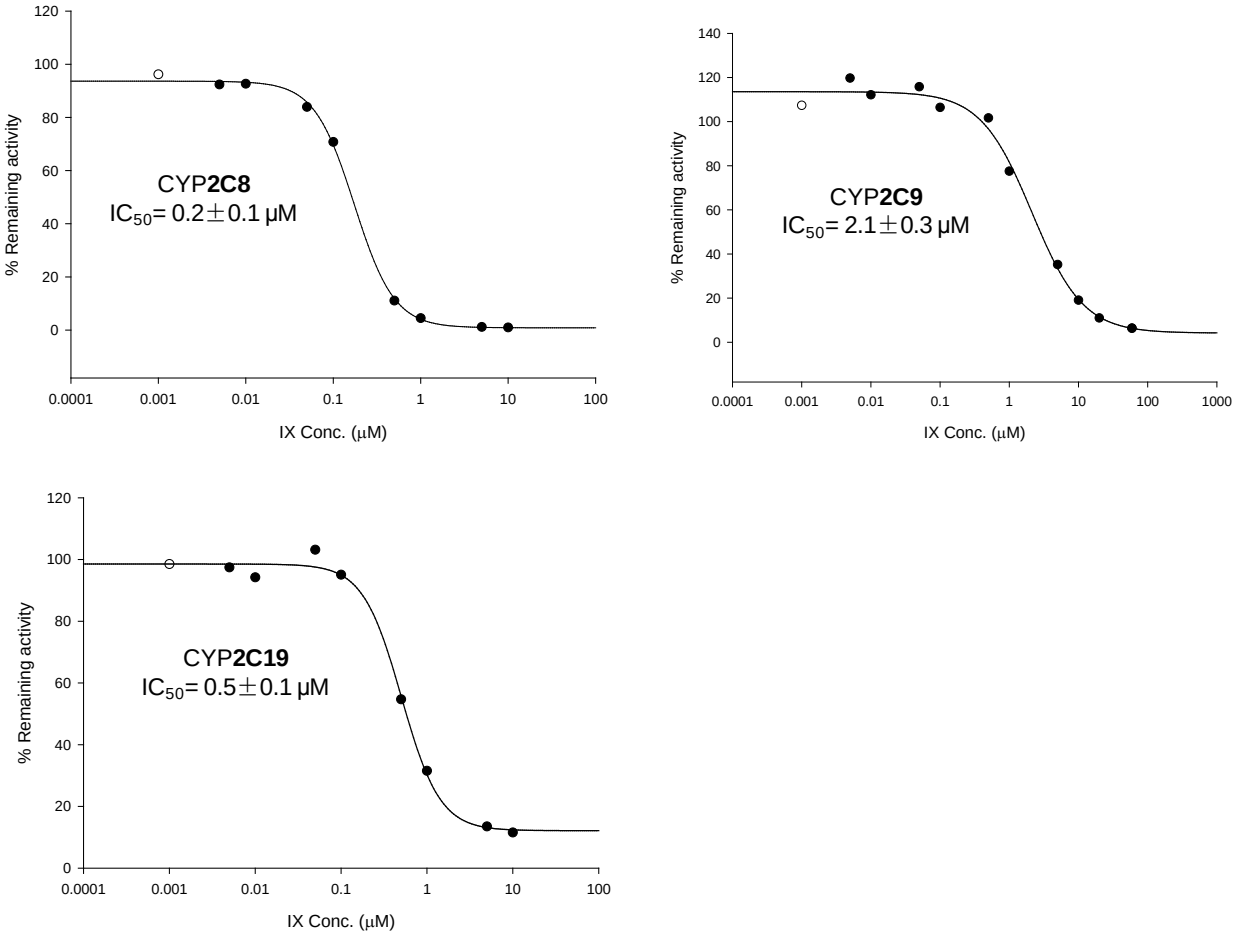


Figure 29. IC_{50} curves for IX inhibition of CYP2C8, CYP2C9 and CYP2C19.

TABLE XI

IC_{50} VALUES FOR THE INHIBITION OF SPECIFIC CYTOCHROME P450 ISOFORMS BY A HOP EXTRACT AND HOP PRENYLATED FLAVONOIDS

Compound	CYP1A2	CYP2C8	CYP2C9	CYP2C19
Hops	9.4 ± 0.6[1]	0.8 ± 0.1	0.9 ± 0.1	3.3 ± 0.7
IX	ND[2]	0.2 ± 0.1	2.1 ± 0.3	0.5 ± 0.1
8-PN	1.1 ± 0.2	0.6 ± 0.1	1.1 ± 0.2	0.4 ± 0.1
XN	ND	1.1 ± 0.1	3.3 ± 0.4	ND
6-PN	ND	1.9 ± 0.1	5.9 ± 0.7	ND

1. Data expressed as mean ± SD.
2. ND: Not determined since ≤ 50% inhibition at 10 μM.

As expected based on the preliminary screening, the hop extract showed strong inhibition of CYP2C isoforms with IC_{50} values of of 0.8 ± 0.1 µg/mL and 0.9 ± 0.1 µg/mL for inhibition of CYP2C8 and CYP2C9, respectively. Inhibition of CYP2C19 was much weaker with an IC_{50} value of 3.3 ± 0.7 µg/mL. Besides inhibition of the CYP2C family, the hop extract moderately inhibited CYP1A2 with an IC_{50} value of 9.4 ± 0.6 µg/mL. Consistent with the hop extract inhibition studies, isolated prenylated flavonoids showed the most potent inhibition against the CYP2C8 isoform with some compounds such as IX showing inhibition in upper nanomolar range. Examination of results in Table XI indicates that positional isomers (8-PN vs 6-PN) or small structural changes in the molecule (methoxy group in IX vs. a hydroxyl group in 8-PN) have profound effects on the selectivity against particular CYP isoforms. For example, 8-PN is a strong inhibitor of CYP2C19 (0.4 ± 0.1) while 6-PN showed very low activity (5.9 ± 0.7). Similarly, IX is poor inhibitor of CYP1A2 while 8-PN is a moderate inhibitor of this isoform.

4.4 Discussion

The majority of drug-drug interactions have been found to result from inhibition of cytochrome P450 enzymes, and inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 were found to be the major targets (161, 162). Nevertheless, CYP2B6, CYP2C8 and CYP2E1 should also be evaluated. Therefore in this investigation of potential drug-botanical interactions, all of these cytochrome P450 enzymes were evaluated for inhibition by a hop extract and by hop prenylated flavonoids.

Stevens *et al.* reported that at 10 µM, XN essentially eliminated CYP1A1 and CYP1B1 activity. They also found >90% inhibition of CYP1A2 by IX and 8-PN at 10 µM by measuring

acetanilide 4-hydroxylase activity (88). We found similar inhibition (88.4%) of CYP1A2 by 8-PN but only 48.2% inhibition by IX at 10 μM. In this study, IX inhibition of CYP1A2 was 48% at 10 μM, which differed from the previously reported value (>90% at 10 μM) (88). These conflicting results might result from the use of different enzyme systems and probe substrate selection. In this dissertation, HLMs were used as the model system and phenacetin as the probe substrate for CYP1A2, while cDNA-expressed human CYP enzymes and acetanilide were used by Stevens *et al.* (14). The HLM model system represents the most appropriate experimental system for the evaluation of inhibitory potential since it provides a more complete system to assess any interactions between the substrate, its metabolites and inhibitors in the presence of the full complement of CYP isoforms. For example, Stevens *et al.* did not report on the inhibition of the CYP2C family enzymes by hops and hop prenylated flavonoids, even though the CYP2C family contributes to the metabolism of ~30% of all drugs.

If recombinant enzymes were used instead of HLMs, products from other isoforms may be missed. In this case, IX was metabolized not only by CYP1A2 but also CYP2C8 (149) so using recombinant enzymes to evaluate the inhibition of IX on CYP1A2 could lead to over prediction of inhibitory effects. Another advantage of using HLM is that the other proteins involved in catalysis (NADPH/P450 reductase and cytochrome b_5) are present at ratios presumably appropriate to the *in vivo* situation.

It has been known that HLMs can influence both CYP and non-CYP inhibition activities. Among the non-CYP inhibition effects, protein binding and non-specific lipid binding properties can influence the free concentration of a test compound and thereby alter the apparent IC_{50} values (163). HLMs with a protein concentration of only 0.1 mg/mL were used in the

experiments reported in this dissertation which minimizes the non specific binding. Therefore, the IC_{50} values that were determined should be more accurate than if higher concentrations had been used as is sometimes the case.

Polymorphisms of drug metabolizing enzymes and drug-drug interactions have a major impact on the efficacy and safety of some drugs that are substrates of the CYP2C family (164). For example, CYP2C8 has become the second most important isoform for drug-drug and perhaps drug-botanical interactions because it is responsible for the metabolism of statins that are widely prescribed to combat hypercholesterolemia. CYP2C9 is responsible for the metabolism of many clinically important drugs such as anticoagulant drug *S*-warfarin and a number of nonsteroidal anti-inflammatory drugs (165). This study found that a hop extract strongly inhibited CYP2C8, CYP2C9 and CYP2C19 with IC_{50} values of 0.8 μg/mL, 0.9 μg/mL and 3.3 μg/mL, respectively. In the subsequent IC_{50} determination of individual compounds from hops, IX was the strongest inhibitor of CYP2C8, and 8-PN was the most potent inhibitor of CYP2C9 and CYP2C19.

For CYP3A4, two or more substrates may be needed as substrate dependence in inhibitor potency has been shown (166). Inhibition of midazolam 1-hydroxylation was evaluated in this study and showed that the hop extract and its major prenylated flavonoids do not inhibit CYP3A4 activity. These results are consistent with those of Stevens *et al*., who used nifedipine instead of midazolam as a probe substrate. A major disadvantage of using nifedipine is that it is a light sensitive reagent and can spontaneously oxidize.

In this dissertation, a standardized hop extract showed strong inhibition of CYP2C8, CYP2C9 and CYP2C19. Individual constituents of hops were tested, and IX was found to be a

strong inhibitor of CYP2C8 while 8-PN showed strong inhibition of CYP2C9 and CYP2C19. Since 8-PN was used to standardize the hop extract, we can establish a quantitative measure for the potential of the extract to inhibit members of the CYP2C family. A mixture of the 4 major prenylated flavonoids was prepared based on their levels in the hop extract. When this mixture was tested for inhibition of cytochrome P450 enzymes in HLMs, the mixture exhibited almost identical inhibition effects against different CYP isoforms, especially the members of the CYP2C family, as shown in Table X. The prenylated flavonoid mixture showed 92.7%, 76% and 65.5% inhibition against CYP2C8, 2C9 and 2C19 compared with inhibition by the hop extract of 92.7%, 88% and 70% against the same isoforms.

Since the extract used in this study was standardized, we were able to estimate the contribution of the four major flavonoids to the overall activity of the crude extract. As shown in Table X, the four flavonoids account for all of the CYP2C8 and 2C19 activity and for the majority of CYP2C9 activity. In contrast, the four flavonoids only partially contributed to the CYP1A2 and CYP2B6 activity suggesting that other compounds are responsible for the observed effect. However, due to low activity of the crude extract against these two isoforms, identification of active compounds was not pursued. In general, in the absence of any interaction among inhibitors, the contribution of a particular compound to the overall inhibition depends on the ratio $[I]/K_i$ where [I] is inhibitor concentration and K_i its inhibition constant. This analysis suggests that XN by virtue of its high abundance in the extract is the major contributor to the observed inhibition of CYP2C family. This analysis does not preclude that other, heretofore unknown CYP 2C inhibitors may be present in the extract. The significance of these

other potential inhibitors may become important for extracts that contain lower amounts of XN that the one used in this study.

It may be concluded that the hop extract inhibits cytochrome P450 enzymes, especially members of the CYP2C family, and may affect the efficacy and safety of some CYP2C substrate drugs when co-administered. Note that his potential for drug-botanical interaction is primarily due to XN, since its presence in the hop extract was 34%. However, further *in vivo* studies are required to determine the significance of these *in vitro* findings.

CHAPTER 5

MAIN CONCLUSIONS AND FUTURE DIRECTIONS

Botanical dietary supplements have the potential to maintain and or even improve health. In particular, some botanical dietary supplements have the potential to prevent cancer. More than 158 million people are taking botanic dietary supplements every day. Lycopene, the most abundant antioxidant carotenoid in tomato, is a promising dietary supplement for the prevention of prostate cancer. During the past 2 decades, many studies have been carried out to investigate the mechanisms of chemoprevention by lycopene. However, these studies have usually addressed just one aspect such as the change in expression of a protein due to lycopene exposure. As a result, the pleiotropic nature of the chemoprevention properties of lycopene has rarely been assessed during a single experiment or set of experiments.

The iTRAQ quantitative proteomics approach described in this dissertation illustrates how lycopene exerts multiple chemoprotective effects on normal human prostatic epithelial cells which are probably the origin of most prostatic adenocarcinomas. Several proteins were up or down-regulated by lycopene in directions that are consistent with reduction of oxidative stress in these cells. For example, up regulation of phase II enzymes such as GSTP1, which is often silenced in prostate cancer, and GSTO1 and SQR can help prevent cancer initiation by detoxifying potentially carcinogenic electrophiles. Lycopene was found to inhibit proliferation of prostate epithelial cells by down regulating the AKT/mTOR pathway and by up regulating genes that have growth inhibitory effects. Lycopene was shown to induce caspase dependant apoptosis and down regulate several proteins involved in anti-apoptosis process including

HSP27, HSP70, TCTP, and KCIP1. Lycopene was also found to alter several signaling pathways including decreased androgen receptor signaling by down regulating protein DJ 1 and HSP90; enhance TNFα signaling induced apoptosis by down regulating TXNDC17; deactivating MAPK pathway and reducing inflammation by down regulating MIF; and down regulation of the AKT/mTOR pathway to slow down cell proliferation and induce apoptosis. All of these effects of lycopene contribute to the prevention of cancer initiation, promotion, and/or progression. This dissertation shows that lycopene is able to modulate all of these chemoprevention pathways, providing a promising rationale for prostate cancer risk reduction.

Although these data are very promising, these studies should be repeated in the future with prostate primary prostatic epithelial cells from more individuals about whom more information is available. There might be specific populations of men who would benefit considerably from lycopene supplements and others who would receive little or less benefit. Future proteomics studies might be carried out using a newer generation of LC-MS/MS systems such as Orbitrap mass spectrometers that might facilitate the identification of more proteins than was possible in this dissertation.

It is a common misconception that botanical dietary supplementsare automatically safe and beneficial simply because they are “natural.” The safety of botanical supplements depends on many factors, such as the safety of the chemical constitutents, the pharmacological activities of the botanical supplement,, how the supplement was prepared, and the dosage. Therefore, it is of great importance to evaluate safety of botanical dietary supplements. \For example, the hop extract investigated in this dissertation had been botanically authenticated, prepared using good manufacturing practice and was standardized both chemically and

biologically. As part of this dissertation, the hop extract was evaluated for the induction or inhibition of human drug metabolizing enzymes as recommended by the 2006 FDA drug interaction guidance for industry (110).

Over a range of concentrations that included the expected human serum levels and levels at least 10-fold higher, showed little or no induction of human CYP1A2 or CYP3A4 enzymes, which are important for drug metabolism. Therefore, hop dietary supplements are not expected to cause adverse drug interactions due to induction of drug metabolizing enzymes. Next, the hop extract and its constituent major prenylated flavonoids were evaluated for potential to inhibit drug metabolizing enzymes. The hop extract showed strong inhibition of CYP2C8, CYP2C9 and CYP2C19. Individual prenylflavonoids were tested, and IX was found to be a potent inhibitor of CYP2C8 while 8-PN showed strong inhibition of CYP2C8 and CYP2C19. Based on these induction and inhibition data, it may be concluded that hops will not induce CYP1A2 and CYP3A4 *in vitro*, but can will inhibit CYP2C8, CYP2C9 and CYP2C19 which may affect the efficacy or cause side effects of co-administrated drugs that are substrates of the CYP2C family of enzymes.

APPENDIX

										Metabolizing enzymes (pmol/min/10^6 cells)								
BD Lot No.	Donor No.	Gender	Age (years)	Race	Cause of Death	Smoker	Medical History	% Viability Post Thaw-High Viability Recovery	Recovery Post Thaw (Cells/vial x 10^6)	Phenacetin O-Deethylation CYP1A2	Amodiaquine N-Demethylation CYP2C8	Diclofenac 4'-Hydroxylation CYP2C9	Bufuralol 1'-Hydroxylation CYP2D6	Testosterone 6b-hydroxylation CYP3A4	AZT-Glucuronidation UGT2B7	7-Hydroxy-Coumarin (7-HC) Glucuronidation UGT	Fold Induction 3A4	Fold Induction 1A2
178	HH261	F	58	C	Stroke/CVA	Y	Whipples Disease	90%	7.6	40	170	310	27	49	93	420	160	18
267	HFC425	F	63	C	Anoxia 2nd to Cardiovascular	Y, quit after 24	Asthma and Diverticulitis	83%	6.5	14	510	1030	25	89	130	350	47	34
285	HMC451	M	61	C	CVA 2nd to ICH	Y	Hypertension x 5 years Med-compliant, HTN, BPH, High cholesterol	95%	7.7	130	310	270	31	330	390	ND	69	46

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PUBLICATIONS:

Yuan Y, **Qiu X**, Nikolic D, Dahl J, van Breemen RB. Method Development and Validation for UHPLC-MS-MS Determination of Hop Prenylflavonoids in Human Serum. *J AOAC Int* (in press)

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PRESENTATIONS:

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Qiu, X *et al*. (2009) Screening of Botanical Extracts for Ligands to Quinone Reductase-2 Using Ultrafiltration LC-MS. 57th ASMS conference on Mass Spectrometry and Allied Topics.