AN ABSTRACT OF THE DISSERTATION OF

Meltem Yilmazer for the degree of Doctor of Philosophy in Toxicology presented on January 5, 2001. Title: Xanthohumol, A Flavonoid From Hops (Humulus lupulus): In Vitro and In Vivo Metabolism, Antioxidant Properties of Metabolites, and Risk Assessment In Humans.

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Donald R. Buhler

Reported here is an investigation to determine the *in vitro* and *in vivo* metabolism of xanthohumol (XN). XN is the major prenylated flavonoid of the female inflorescences (cones) of the hop plant (*Humulus lupulus*). It is also a constituent of beer, the major dietary source of prenylated flavonoids. Recent studies have suggested that XN may have potential cancer chemopreventive activity but little is known about its metabolism. We investigated the *in vitro* metabolism of XN by rat and human liver microsomes, and cDNA-expressed cytochrome P450s, and the *in vivo* metabolism of XN by rats. The metabolites and conjugates were identified by using high-pressure liquid chromatography, liquid chromatography-mass spectrometry, and nuclear magnetic resonance. The antioxidant properties of two metabolites and two glucuronides were examined. The possible risk of XN consumption from beer or dietary supplements is discussed. The involvement of metabolites of XN in cancer chemoprevention remains to be established.
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January 5, 2001

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XANTHOHUMOL, A FLAVONOID FROM HOPS (Humulus lupulus):
IN VITRO AND IN VIVO METABOLISM,
ANTIOXIDANT PROPERTIES OF METABOLITES,
AND RISK ASSESSMENT IN HUMANS

by

Meltem Yilmazer

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Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Meltem Yilmazer, Author
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CONTRIBUTION of AUTHORS

Dr. Donald R. Buhler was instrumental in the preparation of all manuscripts (Chapters 2, 3, 4, 5, 6, and 7) and was responsible for their submission. Dr. Buhler provided advice and expertise in experimental design of the studies in this thesis. Dr. Jan Stevens and Dr. M.L. Deinzer trained me in the analysis of LC/MS and helped in the analysis of NMR (Chapter 2, 3, and 5). Dr. Cristobal L Miranda instructed me in the antioxidation experiments of xanthohumol metabolites (Chapter 6). Dr. Bonnie J. Bailey helped me in the risk evaluation of xanthohumol exposure in Chapter 7.
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I dedicate this thesis to my parents, Tunay and Ahmet,
and to my sisters, Pinar and B. Ege.
Flavonoids represent the most common and widely distributed group of plant phenolics. They are primarily recognized as the pigments responsible for the autumnal burst of hues and the many shades of yellow, orange, and red in flowers and food. More than 5000 chemically unique flavonoids have been identified in plant sources by 1990. As they are ubiquitous and found in fruits, vegetables, nuts, seeds, stems, flowers as well as tea and wine, they are an integral part of the human diet (Bravo, 1998; Middleton and Kandaswami, 1986) and resistant to boiling and fermentation (Kim et al., 1998). Some of the dietary flavonoids and their major dietary source are: genistein (soybeans); quercetin (tea and onions); kaempferol (apples and red wine); epigallocatechin (black tea); luteolin (celery); hesperetin, tangeretin, and naringenin (citrus fruits); and cyanidin (red wine, red berries, red cabbage) (Dragsted et al., 1997).

Flavonoid levels vary greatly even between cultivars of the same species. For example, the formation of flavonoid glycosides greatly depends on light; therefore, the highest concentrations of these compounds are found generally in leaves and outer parts of plants. The presence of flavonoids in food plants is largely influenced by genetic factors and environmental conditions. Other factors, such as germination, degree of ripeness, variety, processing, and storage also
influence the content of flavonoids. Because of all these reasons and dietary habits such as consumption amounts, it is hard to find accurate information on the dietary intake of flavonoids in humans (Bravo, 1998). Depending on dietary habits and/or countries, the daily human intake has been estimated between 3 and 70 mg (Manach et al., 1997). However, it is difficult to compare data within the literature, owing to the lack of agreement on an appropriate method to analyze the different types or families of flavonoids. As a result, information in the literature on the content and composition of flavonoids in plant foods is not only incomplete, but sometimes also contradictory and difficult to compare (Bravo, 1998).

The common structure of flavonoids (Figure 1.1) is that of diphenylpropanes (C₆-C₃-C₆) consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle. They occasionally occur in plants as aglycones, although they are most commonly found as glycoside derivatives.

![Figure 1.1. Basic structure of flavonoids.](image)

Among 13 different flavonoid classes, flavones (e.g. apigenin, luteolin, diosmetin), flavonols (e.g. quercetin, myricetin, kaempferol), and their glycosides are the most common compounds (Middleton and Kandaswami, 1986; Bravo, 1998) (Table 1.1).

The distribution of prenylated flavonoids is restricted to higher plants, and moreover, limited to a few plant families. Over 700 prenylated flavonoids have
been reported in the literature, which is amazing considering their limited distribution in nature. Three of the most important plant families accumulating prenyl flavonoids are the Leguminosae, Moraceae and Asteraceae (Barron and Ibrahim, 1996).

Table 1.1 Classification of Some Food Flavonoids and Basic Structures.
Prenylated flavonoids are often accumulated in the roots as aglycones. Unlike the glycosides of quercetin and kaempferol found in many fruit and vegetables, prenylated flavonoids are not well represented in the human diet, especially not in terms of quantity. Presumably the most important dietary source of prenylated flavonoids is hopped beer. Other dietary sources of prenylated flavonoids include licorice roots (Glycyrrhiza glabra), French beans (Phaseolus vulgaris) and soybeans (Glycine max), but no reports on their levels are available (Stevens et al., 2000a).

Xanthohumol (XN) (Figure 1.2) is a prenylated chalcone flavonoid. Unlike other flavonoids, chalcones lack the heterocyclic C ring (Bravo, 1998).

Figure 1.2. Chemical structure of xanthohumol.

Biogenetically, the A ring usually comes from a molecule of resorcinol or phloroglucinol synthesized in the acetate pathway, whereas the ring B is derived from the shikimic acid pathway (Figure 1.3) (Craig, 1999; Bravo, 1998; Heller, 1986). Power, Tutin and Rogerson were the first named XN in 1913. It is an orange yellow substance with molecular weight 354 and has a melting point at 172°C. XN is insoluble in water and in petroleum ether (Verzele et al., 1957).
XN is the principal flavonoid of hop cones and synthesized in glands located in the female inflorescences and to a lesser extent in glands on the bottom side of young leaves. The hop plant (*Humulus lupulus* L., Cannabinaceae) is a dioecious twining perennial which is cultivated widely throughout the temperate zones of the world for its female inflorescences (commonly referred to as ‘hops’), which are used in the brewing industry to add bitterness and aroma to beer. XN reported at concentration of 0.69 mg/L (1.95 μM) in beer and the total prenylated flavonoid content of beer could be as high as 4 mg/L, or approximately 11 μM (based in the molecular weight of XN) (Stevens et al., 1999 and 2000b).

**Biological Effects of Flavonoids and Xanthohumol**

Flavonoids are essential to plant physiology for their contribution to plant morphology. They are involved in growth and reproduction and provide plants with resistant to pathogens and predators (by acting as phytoalexins or by increasing food astringency, thus making food unpalatable), they protect crops from plague and preharvest seed germination, and act as antioxidants, enzyme inhibitors, precursors of toxic substances, and pigments and light screens (Bravo, 1998).
Figure 1.3 Shikimic acid metabolism.
The effects of flavonoids on mammalian enzyme systems have been demonstrated in many in vitro experiments. Some of these enzymes or enzyme systems are: histidine decarboxylase, DOPA decarboxylase, hyaluranidase, lactic dehydrogenase, pyruvate kinase, catechol-O-methyltransferase, lens adolase reductase, protein kinase C, protein tyrosine kinase, phospholipase A₂, adenosine triphosphatase, phospholipase C, ornithine decarboxylase, amylase, sialidase, adenylate cyclase, cyclic nucleotide phosphodiesterase, RNA and DNA polymerase, topoisomerase, reverse transcriptase, glutathione S-transferase (GST), epoxide hydrolase, glyoxalase, lipoxygenase and cyclooxygenase, aromatase, and xanthine oxidase (Hollman and Katan, 1997; Middleton and Kandaswami, 1986). These enzymes are involved in important pathways that regulate cell division and proliferation, platelet aggregation, detoxification, and inflammatory and immune response (Hollman and Katan, 1997).

Recently, it has been hypothesized that the antioxidant properties of flavonoids may protect tissues against oxygen free radicals and lipid peroxidation. Oxygen free radicals and lipid peroxidation might be involved in several pathological conditions such as atherosclerosis, cancer and chronic inflammation (Hollman and Katan, 1997). It is generally agreed that flavonoids possess both excellent iron chelating and radical scavenging properties. There is much discussion and contradiction regarding their relative antioxidant properties and their structure-activity relationships (SAR) (van Acker et al., 1996). Quercetin, a flavonoid found in many fruits and vegetables, prevents oxidation of low density lipoproteins (LDL) in vitro. As oxidized LDL has been found in atherosclerotic lesions of humans, quercetin may contribute to the prevention of atherosclerosis. Genistein, an isoflavone found in soybeans, has been attracting much attention as a possible anticancer agent. It has a potent growth inhibition against in vitro human breast carcinoma cell lines and rats treated with 7, 12-dimethylbenzanthracene (DMBA) or N-nitroso-N-methylurea (NMU) (So et al., 1996; Fritz et al., 1998). The anticancer activity of quercetin also has been studied extensively, and its growth-inhibitory activity has been shown in vitro in human breast cancer cells,
colon cancer cells lines, a lymphoblastoid cell line, acute lymphoid and myeloid leukemia cell lines, and squamous cell carcinoma cell lines. Naringenin, a flavonoid present in grapefruit juice as its glycoside naringin, had also inhibitory effects on growth of human breast cancer cells \textit{in vitro} (So \textit{et al.}, 1996).

Over the years, a recurring suggestion has been that hops have a powerful estrogenic activity. Hop baths have been used for the treatment of gynaecological disorders and, in earlier days when hops were picked by hand, menstrual disturbances amongst female pickers reportedly were common. The estrogenic activity of hops was attributed to XN but without any evidence. As hops are added into beer for aroma, it is thought that beer may also be estrogenic, too (Milligan \textit{et al.}, 1999; Fenselau and Talalay, 1973). However, further investigations have shown that XN was not estrogenic and that the estrogenic activity of hops is attributable to 8-PN (8-prenylnaringenin) (Milligan \textit{et al.}, 1999).

There are conflicting reports concerning the relationship between beer consumption and cancer risk. For example, Riboli \textit{et al.} (1991) have reported that beer consumption is not associated with colon cancer, but Kato \textit{et al.} (1990) have shown that beer drinkers have an increased risk to colorectal cancer. An inverse association between moderate beer consumption and endometrial cancer was suggested in another study. However, a positive relationship between beer consumption and lung cancer was suggested by Potter \textit{et al.} (1992). It has been shown that beer can inhibit the mutagenicity of Trp-P-2(NHOH), a proximate form of a mutagenic heterocyclic amine present in cooked food (Arimoto-Kobayashi \textit{et al.}, 1999). Further studies with nonvolatile components of beer and an extract of hops have the antimutagenic activity, but ethanol itself at its concentration in beer does not have this activity. These observations indicate that the substances derived from the fermentation process and/or raw plant materials of beer must be responsible for the antimutagenic activity (Arimoto-Kobayashi \textit{et al.}, 1999). In all these biologic effects alcohol and other constituents of beer may play important additive or synergistic roles (Goldberg \textit{et al.}, 1999).
In 1979 a method was patented for treating the skin to protect the skin from erytema-producing sunlight radiation while promoting tanning. This method comprises using as the active sunscreensing ingredient, an ultraviolet radiation absorbing extract of hops (Owades, 1979). A dietary supplement that serves as a general relaxant in adults comprises pharmaceutical grade Kava root extract and 50 mg hops (Bewicke, 2000). XN is patented by US Patent and Trademark Office as it inhibits bone resorption (Tobe et al., 1997). It also exhibits high antifungal activity against Trichophyton spp. (Barron and Ibrahim, 1996; Mizobuchi and Sato, 1984).

It is suggested that prenylation may have a marked influence on the antioxidant activities of chalcones such as XN that is a more powerful antioxidant than alpha-tocopherol (vitamin E) or genistein in preventing LDL oxidation initiated by the metal ion copper (Miranda et al., 2000a). XN also inhibits diacylglycerol acyltransferase (DGAT) activity with an IC₅₀ value of 50.3 μM in rat liver microsomes (Tabata et al., 1997). The growth-inhibitory and cytotoxic activity of XN and other flavonoids from beer were examined in a panel of 60 human cell lines. XN was more growth-inhibitory than other flavonoids in every cancer cell line tested (Miranda and Buhler, unpublished data). In addition, XN exhibited a dose-dependent decrease in DNA synthesis as assessed by the thymidine incorporation assay in human breast cancer (MCF-7) cells. Also it has antiproliferative activity in human colon cancer cells (HT-29) and ovarian cancer cells (A-2780) (Miranda et al., 1999).

Recently, considerable attention has been paid to the ability of flavonoids, including XN, to interact with drug-metabolizing enzymes that are involved in metabolism of various lipophilic xenobiotics in mammals, such as carcinogens, drugs, environmental pollutants and insecticides (Nielsen et al., 1998; Sousa and Marletta, 1985). Flavonoids are reported to have striking effects on the cytochrome-P450 (CYP) dependent monooxygenase system, including the induced synthesis and activation or inhibition of specific P450 isozymes (Henderson et al., 2000; Siess et al., 1992). For example, in in vitro studies a large number of
hydroxylated flavonoids inhibit benzo[a]pyrene (BP) hydroxylation in human liver microsomes. But some flavonoids and other non-hydroxylated analogues act as activators of BP hydroxylation and aflatoxin B1 activation (Buening et al., 1981; Huang et al., 1981). Also, the inducing effects of flavone, flavanolone, and tangeretin on GST activity has been shown (Siess et al., 1992). XN inhibits the catalytic activities of recombinant human CYP1A1, CYP1A2 and CYP1B1 (Henderson et al., 2000) and induces quinone reductase in the mouse hepatoma Hepa 1c1c7 cell lines suggesting that XN also may interact with drug-metabolizing enzymes in vivo (Miranda et al., 2000b).

All of the in vitro studies on flavonoid metabolism can yield useful information on the mechanism of the effect. However, in vitro studies only provide partial results that need to be cautiously interpreted and often require further in vivo studies. These two approaches are complementary and allow a more precise comprehension of the complex mechanisms involved in flavonoid metabolism (Beaune and Guengerich 1988).

The effects of flavonoids on CYPs are complex and may result in pharmacological and toxicological implications in vivo (Siess et al., 1992). Dietary administration of flavonoids to rats is reported to cause significant increases in hepatic CYP catalyzed reactions such as ethoxyresorufin, pentoxyresorufin and ethoxycoumarin dealkylase (Brouard et al., 1988). The synthetic flavonoids, α- and β-naphthoflavone, are well-known inducers and inhibitors of CYP monooxygenase activities, and have been shown to be extensively hydroxylated by CYPs. The polymethoxylated flavone, tangeretin also was demethylated by CYP systems, although the metabolites and their biological effects were not elucidated (Nielsen et al., 1998). The CYP isozyme mainly involved in the metabolism of flavonoids was found to be CYP1A1, but CYP2B and CYP3A forms might also be involved in tangeretin metabolism (Nielsen et al., 1998).

The principal beneficial function of CYPs is to convert chemicals, including XN, into derivatives that can be easily eliminated from the body. This generally occurs as a result of CYP-mediated oxidations of hydrophobic substances followed
by conjugation reactions. But it is also known for many foreign compounds that CYP metabolism results in production of "activated" metabolites. In addition to this, interspecies variability in metabolism, marked intraspecies variability, frequently referred to as drug oxidation polymorphisms, occurs in virtually all mammals examined to date (Gonzalez and Gelboin, 1993). Worries about the mutagenicity of flavonoids in bacterial systems has triggered much research. However, mutagenicity resulting from flavonoid treatment in vivo in mammals was never found (Hollman and Katan, 1997; Aeschbacher et al., 1982).

In addition to in vitro and in vivo studies, epidemiological studies also suggest that consumption of flavonoids from fruit and vegetables may have protective effect against some diseases. The intake of flavonoids is inversely associated with the risk of coronary heart disease in elderly men and protects against stroke (Keli et al., 1996). The epidemiologic data also supports the observation that Asian women consuming a diet high in soy are less susceptible to mammary cancer, but it is not known if this protection occurs as a consequence of prenatal genistein exposure only or postnatal genistein exposure only or if exposure during the complete perinatal period is necessary for protection (Fritz et al., 1998).

Following the discovery about naringenin, a flavonoid in grapefruit, that alters the pharmacokinetics of a variety of drugs by inhibiting CYP enzymes, many researchers focused more attention on the necessity of understanding the fate of flavonoids in the body and flavonoid-drug interactions (Bailey et al., 1998; Fuhr and Kummert, 1995).

Bioavailability of Flavonoids

The bioavailability of flavonoids influences their nutritional significance and potential systemic effects depend largely on their behavior in the digestive tract. Despite their growing importance, little is known about the absorption of flavonoids in the gastrointestinal tract, and whether and how much they are retained
in the body after absorption. Most of the flavonoid studies are done \textit{in vitro} or in animals and very limited research has been done in humans (Bravo, 1998).

Both \textit{in vivo} and \textit{in vitro} studies using polyphenolic compounds with different chemical structures and solubilities illustrated their varying susceptibility to digestion, fermentation and absorption within the gastrointestinal tract. In absorption and metabolism of food flavonoids, the chemical structure of flavonoids plays an important role. The degree of glycosylation/acetylation, their basic structure, conjugation with other phenolics, molecular size, degree of polymerization, and solubility determine their fate in the body (Bravo, 1998).

Absorption of flavonoids from the diet was long considered to be negligible, as they are bound to sugars as glycosides (with the exception of catechins). Only free flavonoids without a sugar molecule were considered to be able to pass the gut wall, and no enzymes that can split these predominantly β-glycosidic bonds are secreted into the gut or present in the intestinal wall (Holiman and Katan, 1997). However, recently it has been shown that the partial absorption of some glycosides takes place in the upper intestine, probably owing to the action of glycosidases from bacteria that colonize the terminal ileum. Quercetin is found in human plasma as glycosides (Andlauer \textit{et al.}, 2000; Bravo, 1998; Paganga and Rice-Evans, 1997). Also it is known that humans absorb appreciable amounts of unhydrolyzed glycosides and that absorption occurs in the small intestine. After consumption of a single portion of onions or apples, a considerable fraction of the absorbed quercetin is present in plasma throughout the day as is indicated by its elimination half-life of 24 h (Hollman \textit{et al.}, 1997; Hollman and Katan, 1997).

Although evidence of the absorption and metabolism of polyphenols in the gut exists, less is known about the efficiency of such uptake and the stability of flavonoids or their conjugates and derivatives in the body. Animal studies with radiolabeled flavonoids and human studies indicate that only partial absorption takes place (Bravo, 1998). For example, some studies suggest that conjugates of daidzein are more bioavailable than those of genistein in rats, probably because of the greater resistance of the former to degradation by gut bacteria. However, the
reverse may be true for quercetin (King, 1998; Sfakianos, 1997). Another study showed that daidzein and genistein but not their glucosides are absorbed from the rat stomach (Piskula et al., 1999).

Metabolism of Flavonoids

Despite the intensive interest on flavonoids, the metabolism of this class of plant xenobiotics and the biological significance of metabolites have been only briefly investigated (Bravo, 1998). The main organ involved in the metabolism of flavonoids is the liver. Plasma albumin also is certainly involved in flavonoid bioavailability since dietary rutin is recovered in substantial concentration in rat plasma in the form of two conjugated metabolites of quercetin bound to albumin. Since kidney and intestinal mucosa contain the enzymes involved in flavonoid metabolism, the contributions to metabolism by these organs cannot be ruled out (Manach et al, 1995).

The methylation and/or conjugation with glucuronide or sulfate are common flavonoid metabolism pathways (Bravo, 1998). It is known that a rapid glucuronidation of diosmetin takes place and that it circulates as glucuronides in rat blood (Boutin et al., 1993) and glucuronidation is indeed the main metabolic pathway for quercetin and kaempferol (Oliveria and Watson, 2000). It is also suggested that the capacity to form isoflavone sulfates is greater in the human GI tract than in that of the rat (Cimino et al., 1999). These conjugates are excreted in the urine or in the bile. In this case, they also can enter an enterohepatic cycle following their deconjugation by the action of the colonic microflora and their absorption. Alternatively they can be fully metabolized and converted into simple phenolic acids after hydrolysis of their flavone structure mediated by bacterial enzymes (Hollman, 1997; Bravo, 1998).

Recent studies also suggest that the health benefit of flavonoids in food can be due to the antioxidant properties of both the parent compounds or their metabolites. For example, quercetin is conjugated in in vivo, yielding metabolites
that exhibit antioxidant properties (Morand et al., 1998). Also, cyanidin 3-O-β-D-glucoside, a potent antioxidant anthocyanin, was not found in rat plasma after oral administration. However its metabolites were found in plasma suggesting that metabolism may contribute to the antioxidant activity (Tsuda et al., 1999). *Ginko biloba* flavonoids were also detected as metabolites in rat blood, not as intact flavonoids (Pietta et al., 1995).

The aim of the present study is to identify *in vitro* metabolites of XN by rat and human liver microsomes, to determine the absorption and possible metabolism pathways for XN in rats and to investigate the antioxidant property of metabolites against LDL oxidation. Also the risks of flavonoids including XN in the human diet are discussed, as the biological effects are dependent on many factors. It will help further studies to clarify the biological effects of XN, as it may be a chemopreventive drug candidate.
CHAPTER 2

IN VITRO BIOTRANSFORMATION OF XANTHOHUMOL, A FLAVONOID FROM HOPS, (*Humulus lupulus*), BY RAT LIVER MICROSONES

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Abstract

Xanthohumol (XN) is the major prenylated flavonoid of the female inflorescences (cones) of the hop plant (*Humulus lupulus*). It is also a constituent of beer, the major dietary source of prenylated flavonoids. Recent studies have suggested that XN may have potential cancer chemopreventive activity, but little is known about its metabolism. We investigated the biotransformation of XN by rat liver microsomes. Three major polar metabolites were produced by liver microsomes from either untreated rats or phenobarbital-pretreated rats as detected by reverse-phase HPLC analysis. Liver microsomes from isosafrole- and β-naphthoflavone-pretreated rats formed another major nonpolar metabolite in addition to the three polar metabolites. As determined by LC/MS and $^1$H-NMR analysis, the three major polar microsomal metabolites of XN were tentatively identified as: a) 5"'-isopropyl-5"'-hydroxy-dihydrofurano[2"',3"':3',4']-2',4-dihydroxy-6'-methoxychalcone; b) 5"'-2-hydroxyisopropyl-dihydrofurano[2"',3"':3',4']-2',4-dihydroxy-6'-methoxychalcone; and c) a derivative of XN with an additional hydroxyl function at the B ring. The nonpolar XN metabolite was identified as dehydrocycloxanthohumol.
Introduction

The flavonoids are some of the most ubiquitous compounds found in nature. Over 4,000 different naturally occurring flavonoids have been described. Flavonoids are common substances in the daily diet and resistant to boiling and fermentation (Kim et al., 1998). They display a wide range of biological and pharmacological properties (Hollman and Katan, 1998) including antiviral, anticancer, and antioxidant properties (Boutin et al., 1993). Several flavonoids can inhibit or activate cytochrome P450 (CYP) enzyme systems \textit{in vitro} and \textit{in vivo} (Nielsen et al., 1998). Flavonoids, including chalcones, have been shown to inhibit the proliferation and tumor growth of cancer cell lines (Miranda et al., 1999).

The hop plant (\textit{Humulus lupulus} L.) is a dioecious twining perennial. The female inflorescences (hop cones or 'hops') are used in the brewing industry to give beer its characteristic flavor and aroma. Xanthohumol (XN) is the principal flavonoid present in hop cone extracts and has a prenylated chalcone structure (Figure 2.1). XN constitutes 82-89% of the total amount of prenylated flavonoids of different European hop varieties (Stevens et al., 1997). Substitutions on the chalcone structure have a profound influence on the anticarcinogenic effects of these compounds. More specifically, studies have shown that XN is an effective antiproliferative agent in human breast cancer cells (MCF-7), colon cancer cells (HT-29) and ovarian cancer cells (A-2780) (Miranda et al., 1999). Other studies such as inhibition of CYP enzymes (Henderson et al., 2000) and induction of quinone reductase (Miranda et al., 2000) by XN suggest that XN may have promising cancer chemopreventive properties.

Although flavonoids are abundantly present in the human diet and in animal feeds, little is known about their metabolism. Two major sites of flavonoid biotransformation are the liver and the colonic flora (Hollman and Katan, 1998).
Since the CYP superfamily is abundant in the liver and small intestine, they may play a role in the metabolism of flavonoids, including XN. The metabolism of flavonoids such as naringenin, genistein, hesperetin, quercetin, chrysin, apigenin, tangeretin, kaempferol, galangin, tamarixetin, taxifolin, luteolin, myricetin, morin, and fisetin has been investigated in rat liver microsomes, but there is no information about metabolism of prenylated flavonoids (Nielsen et al., 1998; Roberts-Kirchhoff et al., 1999). Thus, in the present study, we examined the biotransformation of the prenylated chalcone, XN, in liver microsomes from rats untreated and treated with various P450-inducing agents, including phenobarbital, isosafrole, and β-naphthoflavone. The metabolites were characterized by HPLC, UV spectroscopy, LC/MS, and 1H-NMR.

Materials and Methods

Chemicals

Xanthohumol, desmethylxanthohumol, dehydrocycloxanthohumol hydrate (DH, also referred to as xanthohumol B), and dehydrocycloxanthohumol (DX, also referred to as xanthohumol C) were isolated and purified from hops during previous work (Stevens et al., 1997; 2000b). Troleandomycin (TAO), formic acid, phenobarbital (PB), β-naphthoflavone (BNF) and β-nicotinamide adenine
dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Company (St. Louis, MO). α-Naphthoflavone (ANF) and isosafrole (ISF) were from Aldrich Co. (Milwaukee, WI). Acetonitrile, ethanol and methanol were HPLC grade from Mallinckrodt Baker, Inc. (Paris, KY). Dimethyl-d₆ sulfoxide (100%) and MgCl₂ were purchased from Cambridge Isotope Laboratories, Inc (Andover, MA) and Kodak (Rochester, NY), respectively. Proadifen (SKF-525A) and 1,2-epoxy-3,3,3-trichloropropene (TCPO) were from Smith Kline Biochem. (Philadelphia, PA) and Pfaltz and Bauer (Stamford, CN), respectively. Polyclonal antibodies against rat NADPH-cytochrome P450 reductase were prepared by Ms. M. C. Henderson according to methods previously described (Ardies et al., 1987; Kaminsky et al., 1981).

Isolation of Rat Liver Microsomes

Sixteen male Sprague-Dawley rats (165-185 g) body weight) were purchased from Simonsen Company (Gilroy, CA). Animals were divided into four groups with four animals in each cage. Microsomes were prepared as described (Williams and Buhler, 1984) from pooled livers of rats that had been treated intraperitoneally with 0.9% NaCl, PB (80 mg/kg), ISF (150 mg/kg), or BNF (40 mg/kg) daily for three days. On the fourth day after exposure, preceded by a 24-h fasting period, the rats were anaesthetized using CO₂ and killed by asphyxiation. The washed liver microsomes, resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM EDTA, were frozen at −80°C before use. Protein concentrations of liver microsomes were determined by Coomassie Plus™ protein assay from Pierce Chemical Co. (Rockford, IL). CYP contents were determined by the method of Omura and Sato (1964).
Xanthohumol Biotransformation by Rat Liver Microsomes

A typical 0.5 ml biotransformation incubation mixture consisted of 0.5 mg protein, 0.1 M Tris-HCl, pH 7.4 containing 10 mM MgCl₂ and 100 μM XN (dissolved in 3 μl ethanol) as substrate. The reaction was initiated by adding 2 mM NADPH. Incubations were carried out at 37°C for 60 min with continuous shaking in a Dubnoff incubator. Control incubations were performed without the addition of NADPH or without microsomes. Reactions were terminated by adding 1.5 ml ice-cold methanol, followed by centrifugation at 4°C. The supernatants were evaporated to dryness under nitrogen gas. The residues were redissolved in 100 μl 75% CH₃CN containing 1% formic acid, and subsequently analyzed on the same day by HPLC for XN metabolites. All experiments were carried out in duplicate. One set of experiments was done with ethanol alone or without microsomes.

Optimization of Protein and Substrate Concentrations for Xanthohumol Biotransformation

To obtain an appropriate concentration of microsomes for biotransformation, incubations were performed using the following microsomal protein concentrations: 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00 mg/ml. Different concentrations of XN (10, 50, 100, 200, 400, 600, or 800 μM) also were used to obtain the optimal concentration for metabolite production.

Time Course of Biotransformation of Xanthohumol

To study the biotransformation of XN with respect to time, a slightly modified procedure was used. Liver microsomes from ISF-induced rats were used in these experiments so that all four major XN metabolites were formed. Incubations in a final volume of 250 μl were used and reactions were stopped at 0, 10, 20, 30, 40, 50, and 60 min by adding ice-cold MeOH. The samples were
centrifuged and the supernatants evaporated to dryness. HPLC analysis was conducted as described below.

**Incubations with CYP Inhibitors**

Various CYP inhibitors were included in the incubations to give some insight into the identity of the specific enzymes involved in XN biotransformation. Mixtures were incubated at 37°C for 30 min with one of the following inhibitors, with the target enzyme and the indicated final concentration in parenthesis: SKF525 (0.5 mM; most CYPs), ANF (0.1 mM; CYP1A), TAO (0.5 mM; CYP3A) and TCPO (0.1 mM; epoxide hydrolase) prior to addition of XN. Inhibitor concentrations for these experiments were chosen according to those used in previous studies (Lewis, 1996). As these inhibitors and substrate were prepared in 3 μl EtOH, an additional control incubation was included with only EtOH for each incubation to correct for any effect of the solvent on the microsomes. Carbon monoxide (100%) and antibodies raised against NADPH-cytochrome P450 reductase also were used to inhibit the microsomal biotransformation. The pretreatment with 100% carbon monoxide and rat reductase antibodies were for 15 and 30 min, respectively, at room temperature prior to the addition of XN. Inhibition of the reaction was expressed as the percentage decrease in metabolite production based on the peak area of the metabolite obtained from incubation with addition of inhibitor compared to that without.

**HPLC and Isolation of Metabolites by HPLC**

A Waters 2690 HPLC system with a 996 diode-array detector and a 4 μm Nova-Pak C\textsubscript{18} column (3.9x150 mm, Waters) were used to separate metabolites. The column temperature was thermostatically maintained at 35°C. Detection was at 368 nm, with simultaneous scanning from 230 nm to 400 nm. Samples dissolved in 100 μl 25% aqueous acetonitrile containing 1% formic acid were analyzed using
a slight modification of the method of Nielsen et al., 1998. Metabolites were eluted with acetonitrile and water containing 1% formic acid at a flow rate of 0.8 ml/min. The initial 29% CH$_3$CN was increased to 60% over 18 min and then to 84% over the next 10 min with a linear gradient function. At 34 min, the CH$_3$CN was returned to 29% in 4 min, and the column was equilibrated for 10 min before the next injection. The metabolites were identified by LC/MS and $^1$H-NMR after running a large-scale incubation with 12.1 mg XN and a NADPH generating system with glucose-6-phosphate, glucose-6-phosphate dehydrogenase and collecting the fractions of this incubation by preparative HPLC with an Alltech Econosil C$_{18}$ (250 x 22 mm) column at a flow rate of 11 ml/min. All isolated metabolite fractions were lyophilized and redissolved in 25% CH$_3$CN containing 1% formic acid before LC/MS analysis.

**Liquid Chromatograph/Mass Spectrometry (LC/MS)**

LC/MS was performed with Waters 6000A pumps using a 5 µm C$_{18}$ column (250 x 4.0 mm) at a flow rate of 0.8 ml/min. XN metabolites were separated with a linear solvent gradient starting from 40% CH$_3$CN to 100% CH$_3$CN in 1% aqueous formic acid over 30 min. At 35 min, the % CH$_3$CN was returned to 40% in 2 min, and the column was equilibrated for 15 min prior to the next injection. Mass spectra were recorded on a PE Sciex API III+ triple quadropole mass spectrometer (Ontario, Canada) using atmospheric-pressure chemical ionization (APCI) in positive mode, with an orifice voltage of +55 V, source temperature of 60°C, and scanning from $m/z$ 100 to 450. Samples were introduced by loop injection or by HPLC via the heated nebulizer interface set at 500°C. The multiple ion scan mode was employed for selective detection of metabolites. Daughter-ion scanning in the MS-MS mode was used to obtain structural information. The target gas in the collision cell was argon-nitrogen (9:1) at a density of ca 1.8 x 10$^{14}$ atoms cm$^{-2}$. The collision energy was set at 15 V.
**Nuclear Magnetic Resonance (NMR)**

$^1$H-NMR spectra were recorded in dimethyl-d$_6$ sulfoxide at room temperature on a Bruker DRX 600 spectrometer at 600 and 150.9 MHz. Dimethyl-d$_6$ sulfoxide resonances at 2.5 and 39.51 ppm were used as internal shift references. $^1$H-H COSY was performed using standard pulse sequences.

**Results**

XN biotransformation was investigated by incubation of XN with microsomes from male Sprague-Dawley rats. Four different metabolites were detected and tentatively identified by HPLC, LC/MS, and NMR analyses. Three major polar metabolites (M1, M2 and M3) were found with liver microsomes from either untreated or PB-pretreated rats as detected by HPLC analysis (Figure 2.2A). Liver microsomes from 1SF- and BNF-pretreated rats formed another major nonpolar metabolite (M4) in addition to the other three (Figure 2.2B). The retention times for XN and its metabolites were as follows; XN-17.6 min; M1-9.3 min; M2-11.2 min; M3-15.2 min; and M4-22.0 min. No biotransformation of XN occurred in microsomal incubations that did not contain NADPH (Figure 2.2C).
Figure 2.2 A typical high performance liquid chromatogram of xanthohumol (XN) biotransformation mediated by liver microsomes from untreated (A) or ISF-treated rats (B) with NADPH or without NADPH (C). Metabolite 1 (M1); metabolite 2 (M2); metabolite 3 (M3); metabolite 4 (M4).
The amounts of metabolites produced by rat liver microsomes varied considerably by treatment of rats with different CYP inducers (Figure 2.3). Figure 2.4 demonstrates the formation of XN metabolites from ISF-treated male rat liver microsomes over a 1.0 h incubation period. The ratio of the metabolite formation of all three polar metabolites decreased after 20 min incubation, but the nonpolar metabolite, M4, showed a time-dependent increase.

Figure 2.3. Metabolism of xanthohumol at the end of 60 min incubation by liver microsomes from male rats treated with various inducing agents.
Figure 2.4 Metabolite formation of xanthohumol in control rat liver microsomes. — metabolite 1; ⋅ ⋅ ⋅ metabolite 2; — metabolite 3; — metabolite 4.

Kinetic parameters were calculated according to the Michaelis-Menten equation by non-linear least-squares regression analysis (Powell’s Method). As the absorption coefficients of metabolites were unknown, the calculations were made according to an assumption that the metabolites’ UV absorbance was equivalent to XN. These apparent Km and Vmax values are presented in Table 2.1.
Table 2.1. Apparent kinetic rate constants for metabolism of XN in rat liver microsomes.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ (nmol/min*nmol P450)</th>
<th>$K_{\text{m}}$ (µM)</th>
<th>$r^2$</th>
<th>$V_{\text{max}}/K_{\text{m}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.0072 ± 0.0005</td>
<td>20.16 ± 8.81</td>
<td>0.98</td>
<td>0.0004</td>
</tr>
<tr>
<td>M2</td>
<td>0.0911 ± 0.0077</td>
<td>118.97 ± 38.66</td>
<td>0.99</td>
<td>0.0008</td>
</tr>
<tr>
<td>M3</td>
<td>0.0035 ± 0.0006</td>
<td>246 ± 116.39</td>
<td>0.97</td>
<td>0.00001</td>
</tr>
<tr>
<td>XN</td>
<td>1.46 ± 0.119</td>
<td>407.36 ± 79.48</td>
<td>0.99</td>
<td>0.004</td>
</tr>
</tbody>
</table>

**Effects of CYP Inhibitors**

The results of XN’s biotransformation from the male rat liver microsomal incubations after treatment with various inhibitors are presented in Figure 2.5. Carbon monoxide saturation of rat liver microsomes, which inhibited CYP activity, resulted in a decreased formation of metabolites, M1 (66%), M2 (23%), M3 (64%), and M4 (78%). The biotransformation of XN was altered with the addition of SKF-525A, a multiple type of CYP inhibitor, decreasing formation of M1, M2, M3, and M4 by 66%, 41%, 59%, and 62%, respectively. ANF, a reversible inhibitor of CYP1A, decreased M1, M2, and M4 by 55%, 17%, and 82%, respectively. The effect of ANF on M3 could not be determined since ANF acted as a CYP substrate and gave a metabolite peak at the same retention time as M3. TAO, an inhibitor of CYP3A enzymes in rats and humans, inhibited M1 formation by 55% and other metabolites by an average of 21%. TCPO, an epoxide hydrolase inhibitor, decreased M1, M2, and M3 by 37% and M4 by 15%. Polyclonal antibodies against rat NADPH-cytochrome P450 reductase in the incubations caused a decrease of M1, M2, M3, and M4 by 22%, 3%, 30%, and 33%, respectively.
Figure 2.5. Effect of various P450 inhibitors on xanthohumol metabolites produced by isosafrole-induced rat liver microsomes. CO (carbon monoxide), SKF-525 (proadifen), ANF (α-naphtoflavone), TAO (troleandomycin), TCPO (1,2-epoxy-3,3,3-trichloropropene).
Identification of Metabolites

After preparative HPLC isolation, metabolites were characterized by APCI-LC/MS and \(^1\)H-NMR spectroscopy. The structure of M2 was determined by mass and NMR spectrometry. The UV spectrum showed maximum absorption at 372 nm similar to the chalcone flavonoid XN. Its molecular weight, 370 Da, suggested that it contained an additional hydroxyl group. Upon MS-MS fragmentation of the MH\(^+\) ion, loss of a water molecule, loss of the prenyl moiety, and fission of the OC-C\(_\alpha\) bond (Stevens et al., 1997) were most prominent, giving rise to fragments with \(m/z\) 353 [371-18]\(^+\), 251 [A-ring]\(^+\), 233 [251-H\(_2\)O]\(^+\), 179 [A-C\(_4\)H\(_8\)O]\(^+\), and \(m/z\) 147 [B-ring]\(^+\) (Figure 2.6). Proton NMR analysis of M2 (Table 2.2) showed an OMe resonance at \(\delta\) 3.93 (singlet) and two olefinic protons, H-\(\alpha\) and H-\(\beta\) (broad singlet at \(\delta\) 7.66, integrating for two protons).

Table 2.2. \(^1\)H-NMR data for metabolites of XN [\(\delta_H\) ppm, mult. (J in Hz)]

<table>
<thead>
<tr>
<th></th>
<th>M2</th>
<th>M4=DX</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-(\beta)</td>
<td>7.66 s</td>
<td>7.77 s</td>
</tr>
<tr>
<td>H-(\alpha)</td>
<td>7.66 s</td>
<td>7.77 s</td>
</tr>
<tr>
<td>2,6</td>
<td>7.55 d (8.2)</td>
<td>7.53 d (8.9)</td>
</tr>
<tr>
<td>3,5</td>
<td>6.84 d (8.3)</td>
<td>6.88 d (8.4)</td>
</tr>
<tr>
<td>5'</td>
<td>6.17 s</td>
<td>5.93 s</td>
</tr>
<tr>
<td>4''</td>
<td>2.97 d (9.1)</td>
<td>6.71 d (10.4)</td>
</tr>
<tr>
<td>5''</td>
<td>4.69 t (9.1)</td>
<td>5.48 d (10.0)</td>
</tr>
<tr>
<td>6''-Mes</td>
<td>-</td>
<td>1.47 s (2xMe)</td>
</tr>
<tr>
<td>6'-OMe</td>
<td>3.89 s</td>
<td>3.93 s</td>
</tr>
<tr>
<td>2''-Mes</td>
<td>1.23 s, 1.14 s</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 2.6 LC/MS-MS spectrum of M2 (5''-(2-hydroxyisopropyl)-dihydrofurano[2''',3''';4']-2',4-dihydroxy-6'-methoxychalcone).
The six-proton singlet at δ 1.42 was attributed to the two methyl groups of the prenyl moiety. The B-ring protons, H-2/H-6 and H-3/H-5, appeared as a set of doublets at δ 7.55 and 6.85 (J = 10 Hz). The aromatic A-ring proton H-5' gave a singlet at δ 6.17. These resonances clearly indicated that the A-and B-rings of XN had not been oxygenated. The remaining resonances at δ 4.69 (triplet) and δ 2.97 (doublet), which showed interactions with each other in the 1H-1H COSY spectrum, were attributed to a -CH(O)-CH2- spin system, indicating that the prenyl substituent of XN was oxygenated and fixed in a dihydrofuran ring or a dimethylpyrano ring. The latter possibility was ruled out by proton NMR comparison with DH (compound 9 in Stevens et al., 2000b). Metabolite M2 was therefore identified as 5''-(2''-hydroxyisopropyl)-dihydrofuran[2',3'':3',4']-2',4-dihydroxy-6'-methoxychalcone. The proton resonances of the cyclic prenyl moiety were in good agreement with those reported for 5''-(2-hydroxyisopropyl)-dihydrofuran[2'',3''':7,8]-6-prenylnaringenin (compound 4 in Roussis et al., 1987).

M4 was produced only by liver microsomes from ISF- and BNF-treated rats in the presence of NADPH. This metabolite eluted after XN in the HPLC system, indicating that it was a nonpolar metabolite of XN. M4 was characterized as a chalcone since it gave a similar UV spectra to that of XN with a maximum at 374 nm. Its molecular weight, 352 Da, suggested that it was a dehydro derivative of XN. It gave a prominent A-ring fragment [A1H]+ with m/z 233 on collisional activation in LC/MS-MS experiments. The product failed to produce fragment ions associated with loss of water or loss of the prenyl moiety, indicating that the prenyl group of XN was fixed in a dimethylchromeno ring with the hydroxy group at C-4' delivering the hetero atom (Figure 2.7). 1H-NMR analysis confirmed this hypothesis (Table 2.2). The 1H spectrum showed two olefinic protons (H-4'' and H-5'') at δ 6.56 and 5.61 (J = 10 Hz), respectively. The H-α and H-β protons resonated at δ 7.72 (singlet integrating for two protons).
Figure 2.7 LC/MS-MS spectrum of M4 (dehydrocycloanthohumol).
Other signals were attributed to a gem-dimethyl groups (δ 1.42, singlet, 6 protons), H-5' (singlet at δ 6.11), 6'-OMe (δ 3.93), 4-OH (δ 10.11) and to 2'-OH (δ 14.61). These assignments were in agreement with "H-"C interactions observed in the HMBC spectrum of M4. Thus, M4 was identified as 6", 6"-dimethylpyrano-
[2",3":3',4']-2',4-dihydroxy-6-methoxychalcone, trivially named DX (Stevens et al., 1997). The UV, NMR and mass spectral data of M4 were in agreement with those reported for DX (compound 5 in Stevens et al., 1997).

The maximum UV absorption of M1 was at 370 nm and gave a similar UV spectral pattern to XN suggesting that it was a chalcone flavonoid. The LC/MS analysis of M1 showed a prominent MH⁺ ion with m/z 371 suggesting that M1 was a hydroxylated metabolite of XN. MS-MS fragmentation of the MH⁺ ion yielded only two fragment ions. The most prominent of the two had m/z 353 [371-18]⁺ (100%), indicating that a stable product ion is formed after loss of a water molecule. The second daughter ion (m/z 233, 70% intensity) resulted from cleavage of the OC–Cα bond with charge retention on the A-ring. Such A-ring fragment ions are often very abundant in MS-MS spectra of 2'-hydroxychalcones, due to thermal chalcone-flavanone isomerization in the ion source of the instrument and subsequent retro Diels-Alder fission of the γ-pyranone ring (ring C) upon collisional activation (Takayama et al., 1992). Though the MS-MS spectrum of M1 resembled that of xanthohumol B (DH) (Stevens et al., 2000b), M1 was not identical with DH by direct HPLC comparison. The MS-MS pattern permitted us to conclude that the hydroxylation site in M1 was probably on the prenyl moiety, while the stability of the [MH-H₂O]⁺ fragment and exclusion of the structural candidate, DH, suggested that the prenyl substituent was fixed in a dihydrofurano ring. It was assumed that the oxygen at C-4' served as the hetero atom and not 2'-OH, because the high abundance of the RDA fragment indicated the presence of a free 2'-hydroxy function (6'-OH is methylated in XN, with both ortho OH groups alkylated no in-source chalcone-flavanone would take place). With this, one is left with several possible prenyl moieties, two of the most likely being 5'-isopropyl-5'-
hydroxydihydrofurano and 5"-(2-hydroxyisopropyl)-dihydrofurano. The latter structure was assigned as M2 on the basis of MS-MS and $^1$H NMR data (see above), hence M1 was tentatively identified as 5"-isopropyl-5"-hydroxydihydrofurano[2",3": 3',4']-2',4-dihydroxy-6'-methoxychalcone. The position of the hydroxy group incorporated into XN could not be determined by NMR because the amount of M1 isolated turned out to be insufficient.

The characterization of M3 was based on UV and mass spectral data only, as the amount of this metabolite was not sufficient for NMR analysis. The UV spectrum of M3 appeared to be similar to that of XN with a peak maximum at 375 nm suggesting a chalcone-like structure. The molecular weight of M3 was determined to be 370 Da by APCI-MS, indicating that M3 was also a hydroxylated metabolite of XN. MS-MS fragmentation of the MH$^+$ ion resulted in loss of the prenyl group (see peak at $m/z$ 315 [371-56]$^+$ in Figure 2.8), while RDA fragments appeared at $m/z$ 235 and 179 [235-56]$^+$. Loss of 56 mass units (C$_4$H$_8$) indicated that the prenyl substituent had not been oxygenated during incubation with liver microsomes. This is consistent with the metabolite’s failure to produce ‘dehydrated’ ions (e.g. [MH-H$_2$O]$^+$) upon MS-MS spectra of the hydroxyprenyl derivatives, M1 and M2. Neither was the A ring metabolically modified: this piece of information was deduced from the peak at $m/z$ 179, attributable to an RDA fragment ion also observed in MS-MS spectra of XN. Although none of the fragment ions could be related to the B-ring itself because of charge retention on the A-ring, it was concluded from the MS data that the oxygenation site was on the B ring (C-2 or C-3) of XN. A summary of the pathways for metabolism of XN to M2, M3, M4 and M1 with proposed intermediates is shown in Figure 2.9 and 2.10.
Figure 2.8 LC/MS-MS spectrum of M3 (a hydroxyl derivative of xanthohumol).
Figure 2.9 Proposed biotransformation pathway of xanthohumol to M2, M3, and M4 by rat liver microsomes. Reaction steps: a. CYP hydroxylase; b. CYP epoxidase; c. nucleophilic attack at C-2'' (chemical); d. cyclization (chemical); e. nucleophilic attack at C-3'' (chemical); f. cyclization (chemical); g. dehydration (chemical).
Figure 2.10 Proposed biotransformation pathway of xanthohumol to M1 by rat liver microsomes. Reaction steps: a. CYP epoxidase; b. epoxide hydrolase; c. dehydration (chemical); d. keto-enol tautomerism; e. furano ring formation by intramolecular nucleophilic nucleophilic attack at the carbonyl carbon (chemical).
Discussion

In this study, we characterized the *in vitro* metabolism of XN by rat liver microsomes. Untreated male rat liver microsomes converted XN to three major metabolites, namely M1, M2, and M3 (Figure 2.2A). The formation of these metabolites required NADPH and was inhibited by 100% carbon monoxide and SKF-525A, confirming the role of CYP in XN biotransformation by rat liver (Figure 2.5). Liver microsomes from rats pretreated with PB, an inducer of CYP2B1 and CYP2B2 (Lewis, 1996), metabolized XN to the same three major metabolites as found in untreated rats but in slightly different proportions (Figure 2.3). Based on the experiments, it became evident that M2 formation by liver microsomes from untreated rats followed Michealis-Menten kinetics (Table 2.1) as did the production of M1 and M3, but the formation of latter two metabolites by liver microsomes from untreated rats had much less favorable kinetic parameters than that of M2 (apparent $V_{\text{max}}/K_{\text{m}}$ relative ratios of 0.0004 and 0.00001 vs 0.0008).

On the basis of multi-wavelength HPLC, LC/MS analysis and $^1$H NMR analysis, M1 and M2 were both identified as hydroxylated isopropylidihydrofurano derivatives of XN. Metabolite 4 was identified as the dimethylchromeno analog, DX. These metabolites are assumed to be derived from a prenyl epoxide intermediate (Figure 2.9), which is in line with earlier findings that hydroxylated pyrano and furano derivatives are common fungal metabolites of prenylated flavonoids that are thought to be formed via a prenylepoxide intermediate (Tanaka and Tahara, 1997).

LC/MS analysis of the liver microsomal incubations allowed M3 to be characterized as 2- or 3-hydroxyxanthohumol. The formation of M3 is consistent with the observation that flavonoids with a *para*-hydroxy group on the B-ring tend to be hydroxylated by microsomal enzymes to the corresponding catechol (3'-4'-dihydroxylated) structure (Nielsen *et al.*, 1998).
Liver microsomes from rats pretreated with the CYP1A inducers, ISF and BNF (Lewis, 1996), yielded the three same polar metabolites plus an additional nonpolar metabolite, designated M4. LC/MS and NMR analysis showed that M4 was identical to the structure of DX previously reported by Stevens et al., (1997). This metabolite is presumably formed by three consecutive reactions: (1) epoxidation of the prenyl group possibly catalyzed by CYP; (2) nucleophilic attack by O-4’ at the C-3” of the epoxidated prenyl group yielding the cyclic XN derivative, DH (Stevens et al., 2000b); and (3) dehydration (Figure 2.9).

The most significant finding in this study was that the prenyl group in the A ring of prenylchalcones is a major site for hepatic metabolism. The formation of metabolites such as M2 and M4 suggests that modification of the prenyl substituent, induced by epoxidation of the double bond, constitutes a major metabolic pathway of prenylated flavonoids (Figure 2.9), although it has been reported that catechol formation on the B-ring of non-prenylated flavonoids was a major site for CYP hydroxylation (Nielsen et al., 1998).

As shown in Figure 2.9, we hypothesize that the formation of M1, M2, and M4 proceeds via an initial epoxidation reaction involving the prenyl group. As TCPO, an epoxide hydrolase inhibitor, did not result in a substantial inhibition of M1, M2 or M4, it seems likely that microsomal epoxide hydrolase was not involved in the formation of these metabolites. However it is possible that hydrolysis of prenyl epoxide metabolites could be catalyzed by contaminating soluble epoxide hydrolase, an enzyme not inhibited by TCPO (Moghaddam et al., 1996). Alternatively, the epoxide could be opened spontaneously in the absence of enzyme catalytic activity, which gains support from the observation that epoxidation of XN with m-chloro peroxybenzoic acid yielded DH and DX, whereas the parent epoxide could not be isolated or detected (unpublished results). TCPO also caused a small reduction in the formation of M3 suggesting a partial inhibition of the hydroxylating CYP as shown previously by Shimada and Sato, (1979).

As the CYP inhibitors used in this study did not completely inhibit metabolite formation, other rat CYPs or some other oxygenases may be involved in
the epoxidation of XN (Tanaka and Tahara, 1997). Since some of these enzymes are insensitive to the CYP inhibitors used in this study, this could explain why M1, M2, and M4 formation were not inhibited totally.

The amount of XN metabolites produced by rat liver microsomes was influenced by the use of various CYP inducers (Figure 2.3). Liver microsomes of untreated rats gave significantly higher formation of M1 and M2 upon incubations with XN than that seen with microsomes from ISF- and BNF-treated rats. These results suggest that the CYP1A may not be involved in the formation of M1 and M2. M3 was produced in greater amounts by the liver microsomes of ISF-treated rats than all other groups. In addition, M4 was only formed with liver microsomes of ISF- and BNF-treated rats, presumably due to the involvement of the CYP1A enzymes in its formation. Thus, the CYP1A family seems to be more involved in the formation of M3 and M4 than of M1 and M2. Also, the high inhibitory activity (82% inhibition) of ANF, a selective CYP1A inhibitor, towards M4 formation supports the conclusion that M4 formation was catalyzed mainly by CYP1A enzymes.

The formation of multiple XN metabolites points to the involvement of more than one CYP in the biotransformation of this plant flavonoid. The constitutive forms of CYPs could be the major catalysts for the formation of M1, M2, and M3, since all of these metabolites were produced in untreated rat liver microsomes. Studies are currently underway to provide conclusive evidence of the role of individual CYPs in the biotransformation of XN by carrying out metabolism experiments using individual CYPs, such as cDNA-expressed human CYPs.

To determine whether CYP3As were involved in the formation of M1, M2, M3, and M4, the CYP3A inhibitor, TAO, was added to the incubation mixture containing ISF-induced rat liver microsomes. TAO caused only a 55% inhibition of M1 and a 21% inhibition for M2, M3, and M4. This was in agreement with the findings of Nielsen et al., (1998) who showed that TAO does not appreciably inhibit the metabolism of flavonoids. This modest inhibition of metabolite
formation by TAO indicates that CYP3As may not significantly contribute to the biotransformation of XN, with the probable exception of M1 formation.

Demethylation was not observed during the metabolism of XN by rat liver microsomal enzymes. None of the metabolites was identified as desmethyl xanthohumol by LC-MS comparison with an authentic sample isolated previously by Stevens et al., (1997). By contrast, other flavonoids with O-methyl groups have been found to undergo demethylation reactions (Nielsen et al., 1998). The presence of a prenyl group in the A ring may preclude CYP catalyzed O-demethylation of XN, possibly due to limited acceptability of the substrate as a result of steric hindrance.

Flavonoids display a wide range of biological effects including antiallergic, anti-inflammatory, antiviral, and anticarcinogenic properties. They have been used in attempts to treat a variety of human diseases (So et al., 1996). Prenylated and non-prenylated flavonoids are present in the normal diet and in herbal supplements but information on the biotransformation of these compounds is limited. For example, these flavonoids could be metabolized by CYPs to physiologically active metabolites and the biotransformation products could affect the activity of CYPs that are involved in carcinogen activation or detoxification.

Human exposure to XN, the principal flavonoid of hops, would be primarily through the drinking of beer or ingestion of dietary supplements containing hop extracts or of herbal drugs (Miranda et al., 2000). It was shown that XN inhibited bone resorption and subsequently XN has been patented as a drug for osteoporosis treatment (Tobe et al., 1997). XN may be a candidate for cancer chemoprevention, as it has been reported that XN induced quinone reductase, a detoxifying enzyme, in wild type Hepa 1c1c7 cells (Miranda et al., 2000) and inhibited the growth of human MCF-7 breast cancer cells (Miranda et al., 1999). Although XN is known to inhibit CYP1A enzyme activity (Henderson et al., 2000), the formation of M4, presumably catalyzed by CYP1A enzymes in liver microsomes of ISF and BNF treated rats, indicated that the XN concentration used in this study did not appreciably inhibit these enzymes. Interestingly, it was reported that the metabolite
of XN, DX (referred to as M4 in the present study), at 0.1 and 1.0 μM showed significant antiproliferative activity in human breast cancer MCF-7 cells (Miranda et al., 1999).

The present findings may shed a different light on the biological activities of XN and its oxygenated derivatives (notably cancer chemopreventive effects), because some of the oxygenated derivatives that accompany XN in hops and beer as minor flavonoids, now appear to be formed from XN by liver microsome enzymes as well. Further studies are needed to clarify the mechanisms for formation of the various XN metabolites and to determine whether similar metabolites are produced by rats and humans in vivo. The biological activities of these metabolites and their involvement in cancer chemoprevention also remains to be established.

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References


CHAPTER 3

IN VITRO GLUCURONIDATION OF
XANTHOHUMOL, A FLAVONOID IN HOPS AND
BEER,
BY RAT AND HUMAN LIVER MICROSONES

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Abstract

Xanthohumol (XN) is the major prenylated flavonoid of hop plants and has been detected in beer. Previous studies suggest a variety of potential cancer chemopreventive effects for XN, but there is no information on its metabolism. The aim of this study was to investigate in vitro glucuronidation of XN by rat and human liver microsomes. Using HPLC, two major glucuronides of XN were found with either rat or human liver microsomes. Release of the aglycone by enzymatic hydrolysis with β-glucuronidase followed by LC/MS and NMR analysis revealed that these were C-4' and C-4 monoglucuronides of XN.

Introduction

Flavonoids are a group of phenolic compounds that naturally occur in fruits, vegetables, nuts, seeds, flowers, and bark. More than 4000 different flavonoids have been described. They have been considered inert and nonessential for human health; however, in the last few years it has been shown that flavonoids affect a wide variety of biological systems in mammals, exhibiting antioxidant, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic effects [1]. Studies show that several flavonoids can inhibit or activate the cytochrome P450 (CYP) enzyme system in vitro and in vivo [2, 3]. Recently in animal studies much attention has been paid to the antioxidant properties and the inhibitory role of flavonoids in various stages of tumor development. This improved understanding of the biological and pharmacological properties of individual flavonoid compounds has led to the development of flavonoid drugs which contain either naturally occurring flavonoids or their chemically modified derivatives [4].

Hop (Humulus lupulus L.) cones are known not only as brewing materials but also in Europe as a medicinal plant. Even today, hops are used as a tranquilizer in folk medicine [5]. Xanthohumol (XN), which has a prenylated chalcone structure, is the principal flavonoid present in hop flower (cone) extracts [6] and is present in beer [7]. Studies have shown that XN is an effective antiproliferative
agent in human breast cancer cells (MCF-7), colon cancer cells (HT-29), and ovarian cancer cells (A-2780) [8]. It also was shown that XN inhibited bone resorption and has been patented as a drug for osteoporosis treatment [9].

Although flavonoids are abundant, little is known about their fate in animals. Flavonoid biotransformation is primarily catalyzed by the liver and the gut microflora [10]. In a recent study, we showed that XN was metabolized to four different metabolites by liver microsomes from isosafrole (ISF) or β-naphthoflavone (BNF) induced rats [11]. Numerous studies have been published demonstrating that many types of flavonoids are excreted as glucuronides by humans or other mammals [4, 12]. Glucuronidation is the main pathway of the Phase II detoxification processes for most xenobiotics, including flavonoids, and is catalyzed by UDP-glucuronosyltransferases which are membrane-bound and located mainly in the liver endoplasmic reticulum [13]. It is also known that flavonoids may be conjugated in vivo yielding metabolites that exhibit antioxidant properties [1].

The glucuronidation of flavonoids such as diosmetin, quercetin, genistein, rutin, and kaempferol has been investigated in rat and human, but there is no information about glucuronidation of prenylated flavonoids. This study is the first one to demonstrate that XN, a prenylated flavonoid, produced glucuronides by liver microsomes from human or rats untreated or rats pretreated with various CYP-inducing agents, including phenobarbital (PB), ISF, and BNF. The glucuronides were characterized by high-pressure liquid chromatography (HPLC), UV spectroscopy, liquid chromatography-mass spectrometry (LC/MS), and proton-nuclear magnetic resonance (1H-NMR).
Materials and Methods

Chemicals

XN was isolated and purified from hops as described [6]. Formic acid, β-glucuronidase (Type B-1: from bovine liver), PB, BNF and uridine 5'-diphosphogluconuronic acid (UDPGA) were purchased from Sigma Chemical Company (St. Louis, MO). ISF was from Aldrich Co. (Milwaukee, WI). Acetonitrile, ethanol, and methanol were HPLC grade from Mallinckrodt Baker, Inc. (Paris, KY). Dimethyl-d₆ sulfoxide (100%) and MgCl₂ were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA) and Kodak (Rochester, NY), respectively. Human liver microsomes were purchased from Gentest.

Isolation of Rat Liver Microsomes

Sixteen male Sprague-Dawley rats (165-185 g body weight) were purchased from Simonsen Company (Gilroy, CA). Animals were divided into four groups with four animals in each cage. Microsomes were prepared as described by Williams and Buhler (1984) [14] from pooled livers of rats that had been treated intraperitoneally with 0.9% NaCl, PB (80 mg/kg) in NaCl, ISF (150 mg/kg) in corn oil, or BNF (40 mg/kg) in corn oil daily for three days. On the fourth day after exposure, preceded by a 24-h fasting period, the rats were anaesthetized using CO₂ and killed by asphyxiation. The washed liver microsomes, resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1mM EDTA, were frozen at -80°C before use. Protein concentrations of liver microsomes were determined by Coomassie Plus™ Protein Assay from Pierce Chemical Co. (Rockford, IL). P450 contents were determined by the method of Omura and Sato (1964) [15].
**Xanthohumol Glucuronidation by Rat or Human Liver Microsomes**

A typical 0.5 ml incubation mixture consisted of 0.5 mg protein of liver microsomes from untreated or PB-, ISF-, or BNF-treated rats or human subjects, 52 mM HEPES/NaOH buffer-pH 7.4 containing 10 mM MgCl₂, 0.25 mM triton X-100, 52 mM UDPGA, and 100 μM XN (dissolved in 3 μl ethanol) as substrate [12]. The reaction was initiated by adding UDPGA. Incubations were carried out at 37°C for 60 min with continuous shaking in a Dubnoff incubator. Control incubations were performed without the addition of UDPGA or without microsomes. Reactions were terminated by adding 1.5 ml ice-cold methanol, followed by centrifugation at 4°C. The supernatants were evaporated to dryness under nitrogen gas. The residues were redissolved in 100 μl 25% CH₃CN containing 1% formic acid, and subsequently analyzed on the same day by HPLC for XN metabolites. All experiments were carried out in duplicate. Sets of experiments also were done with ethanol alone or without microsomes.

**Time Course of Glucuronidation of Xanthohumol**

Liver microsomes from untreated rats were used in incubations as described above with a final volume of 250 μl. Reactions were stopped at 0, 10, 20, 30, 40, 50, and 60 min by adding ice-cold methanol. The procedure described above was followed to analyze in HPLC.

**Hydrolysis with β-Glucuronidase**

The supernatants evaporated to dryness of fractions or incubation samples were incubated in 50 mM phosphate buffer (pH 5.5) with the presence of 1000 units/ml of β-glucuronidase for 1.5 hr at 37°C with a final volume was 250 μl. Controls were run simultaneously under the same conditions without β-glucuronidase. All reactions were carried out in duplicate. The reactions were
stopped by adding 500 µl of ice-cold methanol and centrifuged for 20 min. The supernatant was analyzed by HPLC.

**HPLC Analysis and Isolation of Metabolites by HPLC**

A Waters 2690 HPLC system with a 996 diode-array detector and a 4 µm Nova-Pak C₁₈ column (3.9x150 mm, Waters) were used to separate metabolites. Detection was at 370 nm. Samples dissolved in 100 µl 75% aqueous acetonitrile containing 1% formic acid were analyzed using a slight modification of the method of Nielsen et al., 1998 [2]. Metabolites were eluted with acetonitrile and water containing 1% formic acid at a flow rate of 0.8 ml/min. The initial 29% CH₃CN was increased to 60% over 18 min and then to 84% over the next 10 min with a linear gradient function. At 34 min, the CH₃CN was returned to 29% in 4 min. The metabolites were identified by LC/MS and ¹H-NMR after running a large-scale incubation with 1.27 mg XN and collecting fractions of this incubation by preparative HPLC with an Alltech Econosil C₁₈ (250 x 22 mm) column at a flow rate of 11 ml/min. All isolated metabolite fractions were lyophilized and redisolved in 25% CH₃CN containing 1% formic acid before LC/MS analysis.

**LC/MS Analysis**

LC/MS was performed with Waters 6000A pumps using a 5 µm C₁₈ column (250 x 4.0 mm) at a flow rate of 0.8 ml/min. XN metabolites were separated with a linear solvent gradient starting from 40% CH₃CN to 100% CH₃CN in 1% aqueous formic acid over 30 min. At 35 min, the % CH₃CN was returned to 40% in 2 min, and the column was equilibrated for 15 min prior to the next injection. Mass spectra were recorded on a PE Sciex API III+ triple quadropole mass spectrometer using atmospheric-pressure chemical ionization (APCI) in positive mode, with an orifice voltage of +55 V, source temperature of 60°C, and scanning from m/z 110 to 800. Samples were introduced by loop injection or by HPLC via the heated
nebulizer interface set at 500°C. After data acquisition, multiple-ion monitoring was employed for selective detection of metabolites. Daughter-ion scanning in the MS-MS mode was used to obtain structural information. The target gas in the collision cell was argon-nitrogen (9:1) at a density of $ca \ 1.8 \times 10^{14}$ atoms cm$^{-2}$. The collision energy was set at 15 V.

**NMR Analysis**

$^1$H-NMR spectra of metabolite G1 and G2 fractions were recorded in DMSO-d$_6$ at room temperature on a Bruker DRX 600 spectrometer at 600 and 150.9 MHz. DMSO resonances at 2.5 and 39.51 ppm were used as internal shift references. $^1$H-H COSY was performed using standard pulse sequences.

**Results and Discussion**

XN is the major prenylated chalcone in hops and beer. It is of general interest to humans because it represents a dietary flavonoid with significant biological activities [8]. In previous work, the oxidative metabolism of XN was studied using rat liver microsomes [11]. The present study deals with glucuronidation of XN investigated by incubation of XN with UDPGA and liver microsomes from rats or humans. The glucuronides were characterized by HPLC, LC/MS and NMR analysis. Untreated male rat liver microsomes metabolized XN to two major and two minor glucuronides, designated G1, G2, G3, G4 (Figure 3.1A). The retention times for XN and its glucuronides were as follows; XN-17.6 min; G1-9.6 min; G2-11.2 min; G3-3.4 min; and G4-5.0 min. Liver microsomes from rats pretreated with PB (an inducer of CYP2B1 and CYP2B2), ISF (a CYP1A inducer) or BNF (a CYP1A inducer) [16], yielded the same four glucuronides. The relative proportions of these glucuronides was not affected by pretreatment of rats with the different CYP inducers which is in agreement with previous studies and which shows that the microsomal UGTs responsible for the conjugation of flavonoids are not inducible by CYP1A and CYP2B inducers [12].
Figure 3.1 A typical HPLC chromatogram of XN glucuronidation mediated by liver microsomes from untreated rats with UDPGA (A) and without UDPGA (B).
Based on integrated peak areas, G1 and G2 represented approximately 89% and 10% of total glucuronides formed in incubations, respectively. G3 and G4, however, only accounted for about 1% of total glucuronides. Human liver microsomes (H056) that were rich in CYP1A2 formed only the G1 and G2 glucuronides. However, human liver microsomes (H112) that were rich in CYP3A4 produced a small amount of the third glucuronide, G3, in addition to G1 and G2. This may be a result of different concentrations of UDP-glucuronosyltransferase isozymes in the two human liver microsomal samples. Control incubations were carried out without UDPGA or microsomes. No glucuronidation of XN occurred in the absence of UDPGA, and neither were glucuronides formed when liver microsomes (rat or human) were absent (Figure 3.1B).

β-Glucuronidase treatment of the mixture of four glucuronides caused a substantial decrease of amounts of G1, G2, G3 and G4 (95%, 91%, 96%, and 92%, respectively) and a 30%-increase of XN. The hydrolytic cleavage experiments with bovine liver β-glucuronidase confirms that metabolites G1 through G4, are glucuronide conjugates. The time course of glucuronidation of XN by untreated male rat liver microsomes showed an increase of glucuronide formation and a corresponding decrease of parent compound, XN, over a 1.0 h incubation period (Figure 3.2).
Figure 3.2 Time course of xanthohumol glucuronides by liver microsomes from untreated male rats as determined by HPLC.  
- glucuronide 1;  
- glucuronide 2;  
- xanthohumol

The treatment of the G1 and G2, obtained by preparative HPLC, with β-glucuronidase led to the disappearance of G1 and G2 and the concomitant appearance of XN (Figure 3.3 and Figure 3.4, respectively).
Figure 3.3 HPLC chromatogram of G1 before (A) and after (B) β-glucuronidase treatment.
Figure 3.4 HPLC chromatogram of G2 before (A) and after (B) β-glucuronidase treatment.
The structure of two major glucuronides, G1 and G2, were identified as monoglucuronides of XN by UV, LC/MS and $^1$H NMR analysis after preparative HPLC isolation of the glucuronides. G1 showed a maximum UV absorption at 370 nm similar to the chalcone flavonoid XN. In LC/MS studies the molecular weight, $Mr\ 530$, of G1 suggested that the conjugate contained a glucuronide group attached to XN (Figure 3.5A). Upon MS-MS fragmentation of the $MH^+$ ion, loss of the prenyl moiety from glucuronide, and loss of glucuronide from parent compound, were most prominent, giving rise to fragments with $m/z\ 475\ [531-56]^+$, and $355\ [531-176]^+$, respectively. The intensity of the fragment with $m/z\ 355$ was greater than of $m/z\ 475$ indicating that C1"'-C2"' cleavage of the prenyl group was more difficult in G1 than in G2 (compare Figures 3.5A and 3.5B). This suggested that in G1 the glucuronide residue was attached to OH-4' because cleavage of the 1"'-2"' bond of the prenyl substituent leaves an Ar-CH$_2$ fragment ion which is stabilized by a free ortho hydroxyl group [6]. Apparently, such a free hydroxyl group was not available in G1, rendering loss of the prenyl substituent less favorable. In G1, the hydroxyl group at C-4' was connected to the glucuronide residue while OH-2' was chelated with the keto function of XN. Further fragments of G1 were observed at $m/z\ 299\ [355-56]^+$ and $179\ [A-C_4H_8]^+$. Proton NMR analysis of G1 showed an OMe resonance at $\delta\ 3.91$ (singlet) and two olefinic protons, H-\(\alpha\) and H-\(\beta\) (broad singlet at $\delta\ 7.71$, integrating for two protons). The B-ring protons, H-2/H-6 and H-3/H-5, appeared as a set of doublets at $\delta\ 7.59$ and $6.85\ (J = 10\ Hz)$. The aromatic ring proton H-5' gave a singlet at $\delta\ 6.47$. Other signals were attributed to 2'-OH ($\delta\ 14.61$), H-2'' ($\delta\ 4.99$), and H-1'' ($\delta\ 3.17$). Compared to XN ($\delta_{H_5}:\ 6.0$) and G2 ($\delta_{H_5}:\ 6.14$), the H-5' resonance of G1 ($\delta_{H_5}:\ 6.47$) resonated at lower field. This strongly suggested that the glucuronide residue was connected ortho to C-5', that is, to the oxygen atom at C-4'.
Figure 3.5 LC/MS-MS spectrum of G1 (A) and G2 (B).
G2 was formed at lower concentrations than G1 by both rat or human liver microsomes. The UV spectrum of G2 showed maximum absorption at 359 nm similar to the chalcone flavonoid XN. Its molecular weight, M_r 530, suggested that G2 contained a glucuronide group attached to XN (Figure 3.5B). MS-MS fragmentation of the MH^+ ion yielded the same fragments as G1. The most conspicuous difference between the fragmentation patterns of G1 and G2 was the relative intensity of the fragment ions. In G2, loss of the prenyl substituent was more favorable than cleavage of the glucuronic acid residue judging from the greater intensity of the [531-56]^+ ion. This suggested that the glucuronic acid molecule was connected to the B-ring hydroxyl at C-4 in G2, leaving the OH-4' available for stabilization of the Ar-CH_2^+ fragment with m/z 475. Proton NMR analysis of G1 showed an OMe resonance at δ 3.85 (singlet) and two olefinic protons, H-α and H-β (broad singlet at δ 7.84, integrating for two protons). The B-ring protons, H-2/H-6 and H-3/H-5, appeared as a set of doublets at δ 7.66 and 7.08 (J = 10 Hz). The aromatic ring proton H-5' gave a singlet at δ 6.14. Other signals were at δ 14.60 (2'-OH) and δ 4.98 (H-2''). The observation that the B-ring protons resonated at 0.1-0.2 ppm lower field in G2 than in G1 suggested the position of the glucuronide residue at OH-4 in G2. The formation of O-glucuronides was expected as shown in other studies for flavonoids [17, 18].

G3 and G4 produced UV maxima at 262/362 nm and 262/358nm, respectively. In LC/MS studies both conjugates gave pseudo-molecular ions with m/z 531, confirming that both minor metabolites were monoglucuronides. β-Glucuronidase-catalyzed hydrolysis of G3, obtained by preparative HPLC, gave rise to a mixture of XN and its flavanone isomer, isoxanthohumol (IX). These data suggest that G3 may be a glucuronide of IX with the glucuronide portion attached to either one of the two free hydroxyls, OH-4 or OH-4' (in the flavanone, IX, OH-2' is fixed in a γ-pyranone system). Since IX can be formed from XN by nonenzymatic isomerization, it is possible that the IX formed by hydrolysis of its glucuronide conjugate could reisomerize to form XN. Alternatively, the IX glucuronide could co-chromatograph with a glucuronide of XN (possibly the 2'-
OH glucuronide) so that both IX and XN are released upon β-glucuronidase hydrolysis.

G4 was obtained in insufficient amounts for further characterization. However, since the UV and MS-MS data showed that it was a monoglucuronide, there was a possibility that G4 was a monoglucuronide of an XN metabolite formed non-enzymatically in these incubation conditions. For example, the involvement of the non-enzymatic oxidations in XN metabolite formation, such as M1, has been shown [11].

Glucuronidation of XN at position C4' (G1) appears to be favored above glucuronidation at C4 (G2) in these experiments. This suggests that the presence of a prenyl group in the A ring does not preclude glucuronyltransferase catalyzed O-glucuronidation of XN as expected due to limited acceptability of the substrate as a result of steric hindrance.

In this study, we have shown that rat and human liver microsomes readily convert XN to glucuronides. Flavonoids are also conjugated in vivo, yielding flavonoid glucuronides that exhibit a variety of biological activities, such as antioxidant [1] and estrogenic activities, and activation of human natural killer cells [19]. Most previous research has not been conducted with non-prenylated flavonoids. It is not yet known if dietary prenylated flavonoids (0.4-4.0 mg/L in hopped beers) are conjugated in vivo and whether or not the resulting glucuronides have biological activity.

Acknowledgements

The authors would like to thank Dr. C.L. Miranda, Dr. M.L. Deinzer and M.C. Henderson for their helpful suggestions and D.A. Griffin, A.W. Taylor and M. Ivancic for their assistance with the LC/MS and NMR experiments. We also acknowledge the financial support of the Turkish Council of Higher Education, the Hop Research Council, and NIH grant No: ES 00210.
References


CHAPTER 4

METABOLISM OF XANTHOHUMOL,
A PRENYLATED FLAVONOID IN HOPS AND BEER,
BY cDNA-EXPRESSED HUMAN CYTOCHROME P450s AND HUMAN
LIVER MICROSOMES

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Abstract

Xanthohumol (XN), a flavonoid found in hops (Humulus lupulus) and beer, has been shown in various in vitro tests to inhibit the growth of human breast, ovarian, and prostate cancer cells and also inhibits a number of carcinogen-activating human cytochrome P450s (CYPs). In this study, the in vitro metabolism of XN by several cDNA-expressed human CYPs and human liver microsomes are investigated using HPLC and LC/MS. cDNA-expressed human CYP1A1, CYP1A2, and CYP3A4 were found to be the most active isozymes with regard to XN metabolism. Human liver microsomes rich in CYP3A4 and cDNA-expressed human CYP1A1 produce the same four metabolites formed by rat liver microsomes presented in our previous study. Human liver microsomes and cDNA-expressed human CYPs also convert XN to six additional metabolites.

Introduction

Flavonoids are a large group of polyphenols that are almost ubiquitously present in the plant kingdom (1). Dietary intake of flavonoids has an inverse correlation with mortality from coronary heart disease and stroke (2) and their intake also is associated with lower incidence rates of cancer (3). The biological effects of flavonoids have been shown in mammals, including antioxidant, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic effects (4). Although flavonoids have been receiving considerable attention from researchers, little is known about their metabolism.

Two major sites of flavonoid biotransformation in animals are the liver and the colonic flora (5). Since the cytochrome P450 enzymes (CYP) are abundant in the liver and small intestine, they likely play a role in the metabolism of flavonoids, including XN (3,6).

The CYPs constitute a family of enzymes that are involved in the metabolism of a large number of endogenous and exogenous chemicals, including flavonoids (7). CYP1A1 is the major CYP involved in the metabolism of
flavonoids, but CYP2B and CYP3A forms also may participate in flavonoid metabolism (6). Although these three CYP families are able to metabolize some endogenous chemicals, they appear to be primarily associated with the metabolism of exogenous compounds (7, 8).

The metabolism of flavonoids such as naringenin, genistein, hesperetin, quercetin, chrysin, apigenin, tangeretin, kaempferol, galangin, tamarixetin, taxifolin, luteolin, myricetin, morin, and fisetin has been investigated in rat or human liver microsomes (3, 6), but there is no information about the fate of prenylated flavonoids in humans.

Xanthohumol (XN) which has a prenylated chalcone structure is the principal flavonoid present in hop (Humulus lupulus L.) flower (cone) extracts (9) and is present in beer (10). Also, XN has been patented as a drug for osteoporosis treatment, as it inhibits bone resorption (11). Studies have shown that XN is an effective antiproliferative agent in human breast cancer cells (MCF-7), colon cancer cells (HT-29), and ovarian cancer cells (A-2780) (12). Other studies demonstrating inhibition of CYP enzymes (13) and induction of quinone reductase (14) by XN suggest that XN may have promising cancer chemopreventive properties.

This study is the first one to demonstrate that XN is metabolized by cDNA-expressed human CYP and human liver microsomes. The characterization of the metabolites were achieved by HPLC and LC/MS analysis.
Experimental Procedures

Materials

XN was isolated and purified from hops as described (9). Formic acid was purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile, ethanol, and methanol were HPLC grade from Mallinckrodt Baker, Inc. (Paris, KY). MgCl₂ were purchased from Kodak (Rochester, NY). Human liver microsomes and cDNA-expressed human cytochrome P450s were purchased from Gentest (Woburn, MA).

In vitro Metabolism by cDNA-expressed human cytochrome P450s and Human Liver Microsomes

A typical 0.25 ml biotransformation incubation mixture consisted of 0.5 mg protein of human liver microsomes or 20 pmole of cDNA-expressed human CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2C19, CYP3A4, CYP4A11, CYP2E1), 0.1 M Tris-HCl, pH 7.4 containing 10 mM MgCl₂ and 100 μM XN (dissolved in 1.5 μl ethanol) as substrate. The reaction was initiated by adding 2 mM NADPH. Incubations were carried out at 37°C for 60 min with continuous shaking in a Dubnoff incubator. Control incubations were performed without the addition of NADPH or with EtOH. Reactions were terminated by adding 1.5 ml ice-cold methanol, followed by centrifugation at 4°C. The supernatants were evaporated to dryness under nitrogen gas. The residues were redissolved in 100 μl 75% CH₃CN containing 1% formic acid, and subsequently analyzed on the same day by HPLC for XN metabolites. All experiments were carried out in duplicate.

HPLC Analysis

A Waters 2690 HPLC system with a 996 diode-array detector and a 4 μm Nova-Pak C₁₈ column (3.9x150 mm, Waters) were used to separate metabolites. The column temperature was thermostatically maintained at 35°C. Detection was
at 368 nm, with simultaneous scanning from 230 nm to 400 nm. Samples dissolved in 100 μl 25% aqueous acetonitrile containing 1% formic acid were analyzed using a slight modification of the method of Nielsen et al. (6). Metabolites were eluted with acetonitrile and water containing 1% formic acid at a flow rate of 0.8 ml/min. The initial 29% CH₃CN was increased to 60% over 18 min and then to 84% over the next 10 min with a linear gradient function. At 34 min, the CH₃CN was returned to 29% in 4 min, and the column was equilibrated for 10 min before the next injection.

**LC/MS Analysis**

LC/MS was performed with Waters 6000A pumps using the same column at a flow rate of 0.8 ml/min and HPLC gradient as described above. Mass spectra were recorded on a PE Sciex API III+ triple quadrupole mass spectrometer using atmospheric-pressure chemical ionization (APCI) in positive mode, with an orifice voltage of +55 V, source temperature of 60°C, and scanning from m/z 100 to 450. Samples were introduced by HPLC via the heated nebulizer interface set at 500°C. The multiple ion scan mode was employed for selective detection of metabolites. Daughter-ion scanning in the MS-MS mode was used to obtain structural information. The target gas in the collision cell was argon-nitrogen (9:1) at a density of ca 1.8 x 10¹⁴ atoms cm⁻². The collision energy was set at 20 V.

**Results and Discussion**

**HPLC Analysis**

Human liver microsomes rich in CYP1A2 or CYP3A4 produced seven major metabolites in addition to several minor metabolites of XN detected by HPLC (Figure 4.1). The proportions of the metabolites were slightly higher in the CYP1A2 enriched microsomal preparation.
Figure 4.1. A typical HPLC chromatogram of XN biotransformation mediated by human liver microsomes rich in CYP3A4.

These metabolites, designated M1, M2, M3, M4, M5, M6, and M7 had retention times at 9.3 min, 11.2 min, 14.5 min, 22.3 min, 9.6 min, 12.4, and 15.0, respectively. Without NADPH, no metabolism of XN was observed which suggested that the production of these XN metabolites were CYP mediated.
cDNA-expressed human CYP1A1 produced seven metabolites of XN, M2-M8 (Figure 4.2). The only difference between the metabolites formed by cDNA-expressed human CYP1A1 and human liver microsomes was the absence of M1 and the addition of M8 at 22.1 min in the latter.

Figure 4.2. A typical HPLC chromatogram of XN biotransformation mediated by cDNA expressed human CYP1A1.
The metabolites formed by cDNA-expressed human CYP3A4 were M1, M2, M3 and two metabolites, M9 and M10, at 16.3 min and 23.7 min, respectively. CYP3A4 involvement in XN metabolism was expected, as CYP3A4 mediates the detoxifying metabolism of numerous exogenous substrates and can be regarded as a major pathway for detoxification in man (7). All other cDNA-expressed human CYPs (CYP1B1, CYP2C19, CYP4A11, CYP2E1) tested failed to produce any significant quantities of XN metabolites under the experimental conditions used (Table 4.1).

Table 4.1 Summary of XN metabolite formation by cDNA expressed human CYP and human liver microsomes (the number of + indicates the relative formation of metabolites).

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
<th>M9</th>
<th>M10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver microsomes rich in CYP1A2</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Human liver microsomes rich in CYP3A4</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
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<tr>
<td>CYP1A1</td>
<td>++</td>
<td>+</td>
<td>++</td>
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<td>+</td>
<td>+</td>
<td>++</td>
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<tr>
<td>CYP1A2</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CYP3A4</td>
<td>+++</td>
<td>+++</td>
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<td>+++</td>
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<td>CYP4A11</td>
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<tr>
<td>CYP1B1</td>
<td></td>
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<tr>
<td>CYP2B6</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>CYP2C19</td>
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<tr>
<td>CYP2E1</td>
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</tbody>
</table>
The UV spectrum of all metabolites, M1-M10, showed maximum absorption at 366-372 nm similar to that of the parent chalcone flavonoid, XN (Table 4.2). This suggested that they all had chalcone structures similar to XN.

Table 4.2 Summary of spectral data for XN and its metabolites, M1, M2, M3 and M4 were also produced by rat liver microsomes as is presented in a previous study (15).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Relevant ions in mass spectrum</th>
<th>uv absorption absorption (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>9.4</td>
<td>370, and 353, 233</td>
<td>370</td>
</tr>
<tr>
<td>M2</td>
<td>11.2</td>
<td>370, and 353, 251, 233, 179, 147</td>
<td>372</td>
</tr>
<tr>
<td>M3</td>
<td>14.5</td>
<td>370, and 315, 179</td>
<td>366</td>
</tr>
<tr>
<td>M4</td>
<td>22.3</td>
<td>352, and 233</td>
<td>366</td>
</tr>
<tr>
<td>M5</td>
<td>9.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M6</td>
<td>12.4</td>
<td>370, and 353, 251, 233, 179, 147</td>
<td>370</td>
</tr>
<tr>
<td>M7</td>
<td>15.0</td>
<td>386, and 371, 353, 299, 179</td>
<td>379</td>
</tr>
<tr>
<td>M8</td>
<td>21.3</td>
<td>-</td>
<td>370</td>
</tr>
<tr>
<td>M9</td>
<td>16.3</td>
<td>-</td>
<td>370</td>
</tr>
<tr>
<td>M10</td>
<td>23.7</td>
<td>-</td>
<td>369</td>
</tr>
<tr>
<td>XN</td>
<td>17.6</td>
<td>354, and 299, 235, 179, 147</td>
<td>370</td>
</tr>
</tbody>
</table>
**LC/MS Analysis**

XN metabolites produced in sufficient quantities were collected from HPLC analysis for subsequent characterization by APCI-LC/MS (Table 4.2). M1 showed a prominent MH\(^+\) ion with \(m/z\) 371 confirming that it was a hydroxylated metabolite of XN. MS-MS fragmentation of the MH\(^+\) ion of M1 yielded \(m/z\) fragment ions with 353 \([371-18]\)^+ indicating that a stable product ion was formed after loss of a water molecule (Figure 4.3A).

![Figure 4.3A](image)

**Figure 4.3.** A) LC/MS-MS spectrum of M1 and its proposed chemical structure. B) LC/MS-MS spectrum of M2 and its proposed chemical structure.
The prominent daughter ion was \( m/z 233 \) (100% intensity) resulted from cleavage of the OC–C\(_{\alpha} \) bond with charge retention on the A-ring. Other fragments were \( m/z 299, 179[A-C_4H_8O]^+, \) and 147[B ring]. The MS-MS pattern let us conclude that the hydroxylation site in M1 was probably on the prenyl moiety. The LC/MS data of M1 was in agreement with that found with M1 formed by rat liver microsomes (15), a metabolite tentatively identified as 5"'-isopropyl-5''-hydroxy dihydrofurano[2"',3'':3',4']-2',4-dihydroxy-6'-methoxychalcone.

A molecular weight of 370 Da indicated that M2 contained an additional hydroxyl group. Upon MS-MS fragmentation of the MH\(^+ \) ion, loss of a water molecule, loss of the prenyl moiety, and fission of the OC-C\(_{\alpha} \) bond (9) were the most prominent fragmentation reactions, giving rise to fragments with \( m/z \) 353 [370-18]\(^+ \), 251 [A-ring]\(^+ \), 233 [251-H\(_2\)O]\(^+ \), 179 [A-C\(_4\)H\(_8\)O]\(^+ \), and \( m/z \) 147 [B-ring]\(^+ \) (Figure 4.3B). M2 had the same properties as the M2 metabolite of XN produced by rat liver microsomes which was identified as 5"'-isopropyl-5''-dihydrofurano[2"',3'':3',4']-2',4-dihydroxy-6'-methoxychalcone in our previous study (15). This XN metabolite had a furan ring probably formed by rearrangement of a CYP catalyzed epoxy intermediate (15). This was in agreement with earlier findings that hydroxylated pyrano and furano derivatives were common fungal metabolites of prenylated flavonoids that were thought to be formed via prenylepoxide intermediates (16).

The molecular weight of M3 was determined to be 370 Da by APCI-MS, suggesting that it also was a hydroxylated metabolite of XN (Figure 4.4A). MS-MS fragmentation of the MH\(^+ \) ion resulted in loss of the prenyl group at \( m/z \) 315 [371-56]\(^+ \), while RDA fragments appeared at \( m/z \) 235 and 179 [235-56]\(^+ \). Loss of 56 mass units (C\(_4\)H\(_8\)) showed that the prenyl substituent had not been oxygenated during incubation with human liver microsomes or cDNA expressed human CYP. The presence of a \( m/z \) 179 ion also indicated that the A ring was not modified. No ion related to the B ring was formed, suggesting the strong possibility that the oxygenation site was on the B ring (C-2 or C-3) of XN. An identical metabolite was formed by rat liver microsomes (15).
Figure 4.4 A) LC/MS-MS spectrum of M3 and its proposed chemical structure.
B) LC/MS-MS spectrum of M4 and its proposed chemical structure.
M4 appeared after XN in the HPLC analysis indicating that it was a nonpolar metabolite of XN. M4 had a molecular weight of 352, suggesting that it was a dehydro derivative of XN. This metabolite gave a prominent A-ring fragment \([\text{A}_{1}\text{H}]^+\) with \(m/z\) 233 on collisional activation in LC/MS-MS experiments (Figure 4.4B). As it did not produce fragment ions associated with loss of water or loss of the prenyl moiety, it was probable that the prenyl group of XN was fixed in a dimethylchromeno ring with the hydroxy group at C-4' delivering the hetero atom. The UV and mass spectral data of M4 was in agreement with those reported for DX (dehydrocycloanthohumol or 6'', 6''-dimethylpyrano-[2'',3'':3',4']-2',4-dihydroxy-6-methoxychalcone, also known as dehydrocycloanthohumol) (9, 15).

M5 was formed only by cDNA expressed CYP1A1. Since it was not adequately separated by HPLC, further investigation was not done on its chemical structure by LC/MS.

M6 had a molecular weight Mr of 370 Da, indicating that it contained an additional hydroxyl group. It gave the same fragment ion parent as M2 suggesting that M6 also may have a furan ring attached to the a ring, but with the hydroxyl group attached to a position than that of M2.

M7 showed a prominent MH\(^+\) ion with \(m/z\) 387 consistent with the addition of two hydroxyl groups to XN. MS-MS fragmentation of the MH\(^+\) ion of M7 yielded \(m/z\) fragment ions with 371[XN+OH], 353 [371-18]\(^+\) indicating that a stable product ion was formed after loss of a water molecule. Prominent daughter ions at \(m/z\) 299 (75% intensity) and 179 [A-C\(_4\)H\(_8\)O]\(^+\) (100% intensity) confirmed that the prenyl substituent was not oxygenated. The positional assignment of hydroxyl groups of M7 would require NMR analysis.

M8 was formed only by cDNA expressed human CYP1A1. Its appearance after XN on HPLC analysis indicated that it was a nonpolar metabolite of XN. The LC/MS data for M8 showed that it was not a hydroxylated or demethylated metabolite of XN, but no further information was available about the structure of M8 due to its low formation.
M9 and M10 were produced only by cDNA expressed human CYP3A4. M10 was a nonpolar metabolite of XN as it was eluted after XN during HPLC analysis. The inadequate amounts of these two metabolites did not let us get more information about their chemical structures. It is not known why these metabolites were formed by the recombinant human CYP3A4 isoform yet were not detected in incubations with human liver microsomes containing high levels of CYP3A4 (Table 4.1).

Our data indicate that XN was a selective substrate of human CYP3A4, CYP1A1 and CYP1A2 enzymes and that hydroxylation was the most frequent metabolic pathway in the metabolism of XN. These results are in agreement with previous studies done with different flavonoids (3,6). Specifically, M1, M2, and M3 formation was mediated by CYP3A4 as they were formed only by cDNA expressed human CYP3A4 and human liver microsomes. The nonpolar metabolite of XN, M4, was produced by c-DNA expressed human CYP1A1 or CYP1A2, and human liver microsomes, suggesting a role for CYP1A2 in XN metabolism. This isozyme exists only in liver and is involved in the metabolism of only a few drugs (17).

Our results also are in agreement with our previous study carried out in rats (15) which indicated a role for CYP2B in the formation of M1 and the involvement of CYP1A1 and CYP1A2 in the formation of M3 and M4. No evidence for demethylation of XN was seen in the present investigation. According to our LC/MS studies, demethylation by CYPs which is very common for methylated flavonoids (6, 18) does not occur at the 6'-OMe position of XN. This may result from the lack of a C ring in the chalcone flavonoid, XN, or possibly as a result of stearic hindrance. But where hydroxylation occurs, it is known that the polarity and planarity of the C ring has minor effects on the degree of hydroxylation (6). Therefore, we think that XN hydroxylation was not affected by the absence of a C ring.

Overall, the results suggested that the major pathways for the metabolism of XN by cDNA expressed human CYPs and human liver microsomes were the
formation of the hydroxylated XN or the cyclization of XN. As it has been known that less polar flavonoids are metabolized faster and more extensively (6), the extensive hydroxylation of XN may lead to the rapid excretion of XN metabolites from the body. CYP metabolism of XN could perhaps result in the formation of compounds that are at least as active as the parent flavonoid as occurs in the metabolism of genistein with the formation of orobol by CYPs (3). CYP metabolism may also result in metabolites with an increased mutagenic activity as seen after the activation of kaempferol by rat liver microsomes, explained, as already hypothesized by MacGregor and Jurd (19), by microsomal hydroxylation of the B-ring in kaempferol, resulting in the formation of quercetin (6). Therefore, it is important to identify the biologic activity of hydroxylated metabolites of flavonoids, including XN.

Moreover, the inhibitory effect of flavonoid aglycones towards several CYP activities might be due to a competitive metabolism of the flavonoids (6) as is seen with naringin from grapefruit juice (20). Flavonoids also may inhibit CYPs 1A1, 1A2, and 1B1, resulting in the decreased level of formation of catechol estradiol products known to damage macromolecules (3) by occupying these CYPs with their own metabolism.

This is the first study showing that XN, a prenylated chalcone flavonoid, was metabolized by cDNA-expressed human cytochrome P450s and human liver microsomes. Further studies are needed to clarify whether the metabolic pathways of XN found in cDNA-expressed human cytochrome P450s and human liver microsomes are identical to those found in vivo in humans. Assuming that the biotransformation pathways elucidated in this study also may occur in vivo, these findings might have a substantial impact on our understanding of the in vivo bioavailability and bioactivity of flavonoids.
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References


CHAPTER 5

IN VIVO BIOTRANSFORMATION OF
XANTHOHUMOL, A CHALCONE FLAVONOID FROM HOPS,
IN RATS

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Abstract

Xanthohumol (XN) is the principal flavonoid of hop cones and also a constituent of beer. Recent studies have suggested that XN may have potential cancer chemopreventive activity. However, its metabolic fate has been never investigated. In this study, we examined the metabolism of XN in male rats after oral administration. Each animal received a single oral dose of XN (50 mg/kg). Urine samples were collected from the treated rats at selected intervals over a period of 24 h and animals were killed at various times to collect plasma. Orally administered XN in rats was rapidly glucuronidated, circulated in the blood as glucuronide conjugates, and then excreted in the urine. Using HPLC, two glucuronides (designated G1 and G4) were found in both plasma and urine samples. Release of the aglycones by enzymatic hydrolysis with $\beta$-glucuronidase followed by LC/MS revealed that both urinary glucuronides were monoglucuronides. In addition, metabolites M1 (Yilmazer et al., 2001a), M11 and M12, and the glucuronide G4 (Yilmazer et al., 2001b) were identified in urine by HPLC and LC/MS. Higher concentrations of the XN glucuronides than the concentrations of the parent XN appeared in urine, but XN excretion increased with time while the glucuronides decreased. While the glucuronides were found in the plasma, only negligible amounts of the parent XN were present in this fraction. Treatment of plasma with $\beta$-glucuronidase yielded the parent XN together with its metabolites M1, M11, M12, and M13. Studies dealing with the biological impacts of flavonoids should take into account their bioavailability and the nature of their circulating metabolites.
Introduction

Flavonoids are a large group of natural polyphenols that are almost ubiquitously present in the plant kingdom. By 1990, more than 5000 chemically unique flavonoids have been identified in plant sources. As they are ubiquitous and found in fruits, vegetables, nuts, seeds, stems, flowers as well as tea and wine, they are an integral part of the human diet (Middelton and Kandaswami, 1986; Bravo, 1998; Ader et al., 2000). Depending on dietary habits and/or countries, the daily human flavonoid intake has been estimated between 3 and 70 mg. Flavonoids are not considered as indispensable as are vitamins (Manach et al., 1997; Pietta 2000). Most plant-derived flavonoids in foods are present as conjugates with a flavonoid aglycone linked to variable sugar moieties by a β-glycosidic bond (Ader et al., 2000), and this affects their uptake, metabolism and subsequent biological activity (Day et al., 1998).

Hop (Humulus lupulus L.) cones are known not only as brewing materials but also as a medicinal plant in Europe. Even today, hops are used as a tranquilizer or bitter stomachic in folk medicine (Tagashira et al., 1997). Xanthohumol (XN) which has a prenylated chalcone structure (Figure 5.1) is the principal flavonoid present in hop flower (cone) extracts (Stevens et al., 1997) and is present in beer (Stevens et al., 1999). Studies have shown that XN is an effective antiproliferative agent in human breast cancer cells (MCF-7), colon cancer cells (HT-29) and ovarian cancer cells (A-2780) (Miranda et al., 1999).

![Figure 5.1. Structure of XN, a prenylated chalcone flavonoid from hops and beer.](image-url)
Also, it was shown that XN inhibited bone resorption and subsequently XN has been patented as a drug for osteoporosis treatment (Tobe, et al., 1997).

Dietary flavonoids are considered to aid in the prevention of coronary heart disease because epidemiological studies have shown an inverse relationship between the intake of dietary flavonoids and such disease (Hollman and Katan, 1997; Miyake et al., 2000). It is also shown that these compounds affect some biological systems in mammals, exhibiting antiinflammatory, antiviral, antiproliferative, and anticancer properties. There is also an increasing interest in the in vivo protective function of natural antioxidants including flavonoids in the diet against oxidative damage caused by free radical species since it has been shown that both the flavonoids and their metabolites may exhibit antioxidant properties (Morand et al., 1998; Tsuda et al., 1999).

Previous research using rat liver microsomes (Yilmazer et al., 2001a) and recombinant human cytochrome P450 isoforms (Yilmazer and Buhler, 2001c) have shown that XN can be oxidized to a number of different metabolites designated M1 to M10. In another study, rat and human liver microsomes were found to convert XN to four different glucuronides designated G1 to G4 (Yilmazer and Buhler, 2001b).

Despite the increasing interest in their biological effects, data available on the absorption and metabolism of flavonoids in humans or experimental animals are still limited. The aim of the present study was to investigate in vivo the metabolism of a prenylated chalcone, XN, in rats. The metabolites were characterized by HPLC, UV spectroscopy, and LC/MS.

Material and Methods

Chemicals

Xanthohumol was isolated and purified from hops during previous work (Stevens et al., 1997; Stevens et al., 2000b). Formic acid, β-glucuronidase (Type
B-1: from bovine liver), and sulfatase were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile was HPLC grade from Mallinckrodt Baker, Inc. (Paris, KY). Dimethyl-d₆ sulfoxide (100%) and MgCl₂ were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA) and Kodak (Rochester, NY), respectively. Propylene glycol (1, 2-propanediol) was from EM Science, Inc. (Gibbstown, NJ).

**Animals and Diets**

Male Sprague-Dawley rats weighing approximately 180 g were fasted overnight and then gavaged with a single dose of 50 mg/kg of XN in less than 1 ml total volume of propylene glycol. Two rats were housed together in metabolism cages in a temperature-controlled room (22°C) with a 12 h dark period.

**Sampling Procedure**

Urine was collected 24 h after the treatment of rats with XN and acidified with acetic acid to prevent losses of XN and its metabolites. At 2, 4, 6, 12, 24 h after XN treatment, rats were anaesthetized using CO₂ and killed by asphyxiation to collect whole blood. Urine samples and plasma that was separated after centrifugation of whole blood were kept at −80°C until use.

**Sampling Treatment**

Sample extractions were done by using a slightly modified method of Sfakianos et al. (1997). Urine and plasma samples were diluted with 10 volumes of 50 mM ammonium acetate buffer, pH 5.0 and extracted by passage over an activated Sep-Pak C₁₈ cartridge equilibrated with 1 ml methanol, 5 ml H₂O, and 2 ml 50 mM ammonium acetate buffer, pH 5.0, respectively. The cartridge then was washed with 3 X 1 ml 10 mM ammonium acetate buffer, pH 5.0. XN and its metabolites were eluted with 2 X 2 ml methanol. The methanol was evaporated
under nitrogen gas at room temperature and the residues reconstituted in 100 µl of 75% of methanol to use either in HPLC analysis or in β-glucuronidase/sulfatase hydrolysis assays. The efficiency of extraction was 75% and it was measured from 0 h urine or plasma samples of rats by adding and extracting the known amount of XN. To determine the nature of the aglycones, dried samples were incubated for 2 h at 37°C in 50 mM sodium phosphate buffer (pH 5.5) containing 1000 units/ml of β-glucuronidase (Boutin et al., 1993) or 0.5 units/ml of sulfatase (Sfakianos et al., 1997) in a final volume of 200 µl.

**HPLC Analysis**

A Waters 2690 HPLC system with a 996 diode-array detector and a 4 µm Nova-Pak C₁₈ column (3.9x150 mm, Waters) were used to separate metabolites. The column temperature was thermostatically maintained at 35°C. Detection was at 368 nm, with simultaneous scanning from 230 nm to 400 nm. Samples dissolved in 100 µl of 75% aqueous acetonitrile containing 1% formic acid were analyzed using a slight modification of the method of Nielsen et al. (1998). Metabolites were eluted with acetonitrile and water containing 1% formic acid at a flow rate of 0.8 ml/min. The initial 29% CH₃CN was increased to 60% over 18 min and then to 84% over the next 10 min with a linear gradient function. At 34 min, the CH₃CN was returned to 29% in 4 min, and the column was equilibrated for 10 min before the next injection. The metabolites were identified by LC/MS and ¹H-NMR after running a large-scale incubation and collecting fractions of this incubation by preparative HPLC with an Alltech Econosil C₁₈ (250 x 22 mm) column at a flow rate of 11 ml/min. All isolated metabolite fractions were lyophilized and redissolved in 25% CH₃CN containing 1% formic acid before injected in HPLC.
**LC/MS Analysis**

LC/MS was performed with Waters 6000A pumps using a 5 μm C\textsubscript{18} column (250 x 4.0 mm) at a flow rate of 0.8 ml/min. XN metabolites were separated with a linear solvent gradient starting from 40% CH\textsubscript{3}CN to 100% CH\textsubscript{3}CN in 1% aqueous formic acid over 30 min. At 35 min, the % CH\textsubscript{3}CN was returned to 40% in 2 min, and the column was equilibrated for 15 min prior to the next injection. Mass spectra were recorded on a PE Sciex API III+ triple quadrupole mass spectrometer using atmospheric-pressure chemical ionization (APCI) in positive mode, with an orifice voltage of +55 V, source temperature of 60°C, and scanning from $m/z$ 100 to 750. Samples were introduced by loop injection or by HPLC via the heated nebulizer interface set at 500°C. The multiple ion scan mode was employed for selective detection of metabolites. Daughter-ion scanning in the MS-MS mode was used to obtain structural information. The target gas in the collision cell was argon-nitrogen (9:1) at a density of ca 1.8 x 10\textsuperscript{14} atoms cm\textsuperscript{-2}. The collision energy was set at 15 V.

**Results**

In addition to XN some of its biotransformation products, the glucuronides designated G1 and G4 (Yilmazer et al., 2001b) and the metabolites M1 (Yilmazer et al., 2001a, 2001c) and a new metabolite designated as M12 were detected by HPLC (Figure 5.2A) in urine samples of rats after single dose treatment of XN (50 mg/kg). The retention times for XN and its metabolites in urine were as follows; XN-17.6 min; G4-5.0 min; M1-9.3 min; G1-9.6 min; and M12-10.7 min. The peak at 15.0 min was a normal background component since it was present in the urine of untreated rats. In plasma only the two glucuronide conjugates, G1 and G4, and a very small amount of the parent XN were detected by HPLC (Figure 5.3A).
Figure 5.2. A typical HPLC chromatogram of urine sample of rats at 12 h after gavaged with a single dose of XN (A), urine sample after β-glucuronidase treatment (B).
Figure 5.3. A typical HPLC chromatogram of plasma sample of rats at 2 h after gavaged with a single dose of XN (A), plasma sample after β-glucuronidase treatment (B).
The enzymatic treatment of the extracted urine samples with β-glucuronidase resulted in a 95% and 100% disappearance of G1 and G4, respectively, and the appearance of M1 together with the new metabolites M11 and M12, and an increase of XN (Figure 5.2B). The enzymatic treatment of the extracted plasma samples with β-glucuronidase resulted 95% and 96% disappearance of G1 and G4, respectively, and increase of a peak at 17.6 min that was identified as XN by LC/MS analysis (Figure 5.3B). Many small metabolite peaks, notably M1 and metabolites M11, M12, and M13, at 9.3 min, 10.5 min, 10.7 min, and 11.5 min also were seen (Figure 5.3B). However, sulfatase treatment did not make a significant change in the metabolite pattern, either in plasma or urine.

None HPLC retention times of the in vivo metabolites matched those of the hop flavonoids isoxanthohumol (IX), dehydrocycloaxanthohumol (DX), dehydrocycloaxanthohumol hydrate (DH), and 6-prenynaringenin. Also, demethylation of XN in the rats did not occur since no 2', 4', 6', 4'-tetrahydroxy-3'-geranylchalcone (TP) was detected.

The three metabolites of XN excreted in urine and the two seen in plasma were characterized by HPLC and LC/MS (APCI). The retention time of M1 excreted in urine and found after β-glucuronidase hydrolysis of urine was 9.3 min. The UV spectrum of M1 shows maximum absorption at 372 nm indicating that it was derived from the chalcone flavonoid XN. Its LC/MS analysis showed a prominent MH⁺ ion with m/z 371 suggesting that M1 was a hydroxylated metabolite of XN (Figure 5.4). MS-MS fragmentation of the MH⁺ ion yielded only two fragment ions. The most prominent of the two has m/z 353 [371-18]+ (100%), indicating that a stable product ion was formed after loss of a water molecule. The second daughter ion (m/z 233, 70% intensity) resulted from cleavage of the OC–Cα bond with charge retention on the A-ring. Such A-ring fragment ions are often very abundant in MS-MS spectra of 2'-hydroxychalcones, due to thermal chalcone-flavanone isomerization in the ion source of the instrument and subsequent retro Diels-Alder fission of the γ-pyranone ring (ring C) upon collisional activation (Takayama et al., 1992). This information was in agreement with the identification
of M1 as a B ring hydroxylation product of XN as shown previously from the \textit{in vitro} metabolite of XN by rat liver microsomes (Yilmazer \textit{et al.}, 2001a) and recombinant human P450s (Yilmazer \textit{et al.}, 2001c).

Figure 5.4. LC/MS-MS spectrum of M1 and its proposed chemical structure.
The UV spectra of G1 and G4 excreted in urine and plasma had a maximum absorption at 245/366 nm and 249/366 nm, respectively, similar to the chalcone flavonoid XN. In LC/MS studies the information about their molecular weight Mr 530 suggest that both of glucuronides contained a single glucuronide group attached to a XN type chalcone (Figure 5.5A and 5.5B). Upon MS-MS fragmentation of the MH⁺ ion, loss of the prenyl moiety from the glucuronide, and loss of glucuronide from the parent compound, were the most prominent ions, giving rise to fragments with m/z 475 [531-56]⁺, and 355 [531-176]⁺, respectively. In G1, the intensity of the fragment with m/z 355 was greater than that of m/z 475 indicating that C1"-C2" cleavage of the prenyl group was difficult. This suggests that in G1 the glucuronide residue was attached to OH-4' because cleavage of the 1"-2" bond of the prenyl substituent leaves an Ar-CH₂⁺ fragment ion which was stabilized by a free ortho hydroxyl group (Yilmazer et al., 2001b). Apparently, such a free hydroxyl group was not available in G1, rendering loss of the prenyl substituent less favorable. In G1, the hydroxyl group at C-4' was connected to the glucuronide residue while OH-2' was chelated with the keto function of XN. Further fragments of G1 are observed at m/z 299 [355-56]⁺ and 179 [A-C₄H₈]⁺. G1 collected from urine and plasma extractions by prep-HPLC was treated with β-glucuronidase and resulted in the appearance of the parent XN compound, in the concomitant disappearance of G1 and the appearance of XN. This result was in agreement with our previous findings from the studies with rat and human liver microsomes that identified G1 as a glucuronide conjugate of XN attached to the 4'-OH position (Yilmazer et al., 2001b).

G4 was seen at lower concentrations than G1 in urine and plasma. The instability and low amounts of G4 present in plasma extracts did not allow us to obtain any information about it by LC/MS. But the MS-MS fragmentation of the MH⁺ ion of G4 isolated from urine extracts gave the same qualitative fragmentation pattern as G1.
Figure 5.5. LC/MS-MS spectrum of G1 and G4 with their proposed chemical structures.
The difference between the fragmentation patterns of G1 and G4 was in the relative intensity of the fragment ions. In G4, loss of the prenyl substituent was more favorable than cleavage of the glucuronic acid residue judging from the greater intensity of the [531-56]$^+$ion.

The LC/MS data for unknown metabolite peaks at 10.7 min, M11, in urine and two unknown peaks at 10.7 min and 11.5 min, M12 and M13, in plasma appeared after β-glucuronidase treatment of samples could not be investigated due to their low concentrations.

The glucuronide conjugates, G4 and G1, were detected in urine samples of rats collected 2 h after oral intake of 50 mg/kg XN and reached a maximum at 12 h after administration (Figure 5.6). The excreted amounts of G1, G4, and XN in urine were estimated at 0.3%, 0.05%, and 0.2% of total administered dose, respectively. The concentration-time curves of M1 in urine couldn't be determined as the concentration of these two metabolites declined below the detection limits during the first 4 h after administration. The two glucuronides and small amount of XN were seen in plasma at 2 h after XN intake and the concentration increased between 2-4 h and decreased after 4 h (Figure 5.7).
Figure 5.6. Cumulative excretion of xanthohumol and glucuronides in urine.
Figure 5.7. Plasma concentrations of xanthohumol and glucuronides by time.

In urine and plasma the estimated amount of G1 was calculated based on the amount of XN released after β-glucuronidase treatment of the isolated glucuronide fraction. However, the amount of G4 was calculated as XN equivalents based on an assumption that XN and G4 had the same absorption efficiency in HPLC. The clearance of XN in urine was 0.14 L/hour according to the formula of Ae24/AUC. However, this value might be overestimated, as the AUC calculated from plasma was missing the early points in data due to the rapid glucuronidation of XN.
XN metabolism by female Sprague-Dawley rats was investigated as a preliminary study to see the gender difference (unpublished data). Female rats excreted the same XN metabolites as male rats but at lower concentrations.

Discussion

During the last years many reports have been published indicating that plant-derived flavonoids are potent oxidants and influence several key enzymes involved in cellular metabolism. However, those results mainly derived from in vitro experiments and recently, metabolism of flavonoids became a matter of interest (Ader et al., 2000). Therefore, the aim of this study was to investigate the metabolism of a prenylated flavonoid, XN, in rats.

After oral administration of 50 mg/kg XN in rats, glucuronide conjugates were present in plasma, but very little of the parent XN. The fact that plasma contained mainly glucuronide conjugates demonstrates effective conjugation of XN. Our findings are in full agreement with those of Pietta et al. (1995), Manach et al. (1997 and 1998), Tsuda et al., (1999), and Ader et al. (2000) who also found that most of the flavonoids examined and were present in the plasma as conjugated forms.

Our results showed that XN was absorbed, formed glucuronides quickly and this extensive conjugate formation led to its rapid excretion in urine. This pattern was similar to that observed for isoflavones in rats where by absorbed flavonoids were not further metabolized, except for the formation of glucuronide conjugates (King and Bursill, 1998). M1 produced by rats was also formed by rat liver microsomes, but M2, M3, and M4, the other three major in vitro metabolites of XN produced by rat liver microsomes (Yilmazer et al., 2001a), were not formed in vivo. Similarly, there was no evidence for the production of XN metabolites M5 to M10 that were previously shown to be formed by recombinant human cytochrome P450 isoforms (Yilmazer et al., 2001c).
The early peak plasma concentration of glucuronides at 2 h after oral administration suggested that the absorption of XN was possibly from the upper gastrointestinal tract as previously proposed for other flavonoids (Ader et al., 2000). The decrease in plasma concentrations between 4 and 24 h after XN administration showed that there was no delay after administration and eliminated the likelihood of metabolites binding to albumin such as seen with catechins (Manach et al., 1999).

LC/MS analysis and the β-glucuronidase treatment of urine and plasma indicated that G4 and G1 were glucuronide conjugates. Furthermore, LC/MS analysis of the compound released upon the enzymatic treatment of G1 showed that this aglycone was XN. The enzyme analysis of G4 isolated from urine showed that G4 was a glucuronide conjugate of another XN metabolite, M1, that was less polar than XN. This is in agreement with the experiments in rats with quercetin that showed the circulation of quercetin in plasma as glucuronide conjugates (Manach et al., 1995). All HPLC, UV spectra, LC/MS analysis of G1 were in agreement with our previous findings, which showed that G1 was identical to the O-glucuronide conjugate of XN at 4′-C (Yilmazer et al., 2001b). The absence of G2, a monoglucuronide of XN at the 4-C position formed by rat and human liver microsomes (Yilmazer et al., 2001b) in the current investigation indicated that glucuronidation of XN at position 4′-C (G1) was favored to the glucuronidation at 4-C (G2) in vivo. This suggested that the presence of a prenyl group in the A ring did not preclude glucuronyltransferase catalyzed O-glucuronidation of the A ring of XN as expected due to limited acceptability of the substrate as a result of steric hindrance.

Demethylation of XN, a common Phase I metabolism pathways of CYP enzymes, was not observed in rats. None of the metabolites were identified as the demethylation product of XN,TP, by LC-MS comparison with an authentic sample isolated previously by Stevens et al. (1997). By contrast, other flavonoids with O-methyl groups have been found to undergo demethylation reactions (Nielsen et al., 1998). The presence of a prenyl group in the A ring may preclude CYP catalyzed
O-demethylation of XN, possibly due to limited acceptability of the substrate as a result of steric hindrance. Also, preliminary studies with female rats in our lab showed a proportional decrease of the same G1, G4, and M1 metabolites in plasma and urine (unpublished data).

An important point to consider in bioavailability studies is whether the biological activity depends on the flavonoid itself or on its metabolites as they circulate in the body or leave the body quickly. Numerous studies have shown the antioxidant activity and various cellular effects of aglycones, but few works have taken into account the fact that the circulating metabolites may have the same effects in different portions. For example, it is shown that quercetin metabolites have a significant protective effect on human or rat lipoprotein oxidation (Morand et al., 1998; Manach et al., 1998). The relative contribution of all the metabolites to the total antioxidant effect is unknown.

Also the combination of XN with alcohol in beer may have an additive effect, as it is shown that nonvolatile components of beer and an extract of hops have the antimitogenic activity, but ethanol itself at its concentration in beer does not have this activity. These observations indicate that the substances derived from the fermentation process and/or raw plant materials of beer must be responsible for the antimitogenic activity reported in beer (Arimoto-Kobayashi et al., 1999). Alcohol also may facilitate XN absorption in the body, as it is a vasodilator agent or interact with flavonoid metabolism by inducing CYP2E1. In all these biologic effects, alcohol and other constituents of beer such as flavonoids including XN may play important additive or synergistic roles (Goldberg et al., 1999). Recently, the increase in plasma vitamin B6, as seen after beer and to a lesser extent after red wine consumption suggested the contribution to a lower cardiovascular disease risk (van der Gaag et al., 2000). The additive effect of flavonoids, including XN, and alcohol in beer needs to be investigated by in vivo studies to determine any biological effects. The bioavailability and tissue distribution of XN in animals and humans, and the biological effects of XN and its metabolites need to be assessed.
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References


CHAPTER 6

ANTIOXIDANT PROPERTY OF XANTHOHUMOL METABOLITES AND GLUCURONIDES, AGAINST RADICAL OXIDATION INDUCED BY 2,2'-AZOBIS-(2-AMIDINOPROPANE) HYDROCHLORIDE

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Abstract

Recently, it has been demonstrated that flavonoids may be metabolized in vivo yielding metabolites that exhibit antioxidant properties and in some cases the activity of a metabolite may be higher than that of parent flavonoid. Xanthohumol (XN), the major prenylchalcone in hops and beer, showed higher antioxidant activity than that of α-tocopherol and the isoflavone, genistein, but lower than the flavonol, quercetin, in inhibiting low-density lipoprotein (LDL) oxidation. In the present study, two cytochrome P450-mediated oxidation products of XN, M2 (5''-(2''-hydroxyisopropyl)-dihydrofurano[2'',3'':3',4']-2',4-dihydroxy-6'-methoxychalcone) and M4 (6'', 6''-dimethylpyrano-[2'',3'':3',4']-2',4-dihydroxy-6'-methoxychalcone), and two glucuronides of XN formed by rat liver microsomes G1 (OH-4' glucuronide) and G2 (OH-4 glucuronide) were examined for their ability to inhibit in vitro oxidation of human LDL mediated by 2,2',-azobis-(2-amidinopropane) hydrochloride (AAPH). The oxidation of LDL was assessed by the formation of thiobarbituric acid-reactive substances (TBARS). At concentrations of 0.5 and 5 μM, the XN oxidation products, M4 and M2, inhibited the oxidation of LDL (0.1 mg protein/ml) induced by 10 mM AAPH. At 1 and 5 μM, the two XN glucuronides, G2 and G1, increased LDL oxidation, based on TBARS formation. These findings suggest that oxidative metabolites of XN like the parent compound, XN, protect LDL from oxidation by peroxyl radicals generated from AAPH. However, glucuronides of XN exerted prooxidants effects on human LDL oxidized by the peroxyl radical generator, AAPH.
Introduction

Oxidative modification of LDL is suggested to play an important role in the development of human atherosclerosis. During the oxidation of LDL, the polyunsaturated fatty acids in the lipoprotein are rapidly converted to lipid hydroperoxides and aldehydic breakdown products (Esterbauer et al., 1992). LDL oxidation also results in the oxidative modification of the apoprotein, which plays a role in macrophage uptake and atherogenesis (Steinberg et al., 1989; Diaz et al., 1997; Esterbauer et al., 1992). Thus, protecting LDL from oxidation by such compounds as flavonoids may be an effective strategy to delay or prevent the progression of the disease (Lebeau, et al., 2000; Diaz et al., 1997). A high intake of flavonoids already has been associated with a lower incidence of cardiovascular diseases (Hertog et al., 1993).

XN is the principal prenylated flavonoid present in hops (Humulus lupulus) (Stevens et al., 1997) and beer (Stevens et al., 1999). Studies have shown that XN is an effective inhibitor of AAPH-induced oxidation of LDL (Stevens et al., 2001) and is a highly active antiproliferative agent against human breast cancer cells (MCF-7), colon cancer cells (HT-29) and ovarian cancer cells (A-2780) (Miranda et al., 1999). In our previous studies, we identified four metabolites of XN (M1 to M4) formed by rat and human liver microsomes (Yilmazer et al, 2001a; Yilmazer et al., 2001c). These metabolites were defined as M1 (5''-isopropyl-5''-hydroxydihydrofurano[2'',3'':3',4'']-2',4-dihydroxy-6'-methoxychalcone), M2 (5''-(2-hydroxyisopropyl)-dihydrofurano[2'',3'':3',4'']-2',4-dihydroxy-6'-methoxychalcone, M3 (B ring-hydroxylated XN), M4 (6'', 6''-dimethylpyrano[2'',3'':3',4'']-2',4-dihydoxy-6-methoxychalcone). Similarly, 4' and 4-monoglucuronide metabolites of XN (G1 and G2) were shown to be formed by human and rat liver microsomes (Yilmazer et al, 2001b).

Recently, it has been demonstrated that flavonoids, such as quercetin, may be metabolized in vivo yielding metabolites that exhibit antioxidant properties. In some cases, the pharmacological activity of a metabolite may be higher than that of parent flavonoid (Morand et al., 1998).
The mechanisms of antioxidant action can include: (1) suppressing reactive oxygen species formation either by inhibition of enzymes or chelating transition metals involved in free radical production; (2) scavenging reactive oxygen species; and (3) upregulating or protecting antioxidant defenses. Flavonoids have been identified as fulfilling most of the criteria described above (Pietta 2000). In the present study, we tested the antioxidant properties of two oxidative metabolites and two glucuronides of XN. To assess the antioxidant activity of the compounds of interest, we evaluated their capacity to inhibit 2,2'-azobis-(2-amidinopropene) hydrochloride (AAPH)-mediated oxidation of LDL by measuring the formation of thiobarbituric acid-reactive substances (TBARS).

**Materials and Methods**

**Materials**

XN was isolated from hops as described by Stevens *et al.* (1997). LDL, prepared from the plasma of healthy volunteers using the method of Chung *et al.* (1986) as modified by Sattler *et al.* (1994), was supplied from Linus Pauling Institute. Rat liver microsomes were prepared as described (Williams and Buhler, 1984) from pooled livers of rats that had been treated intraperitoneally with β-naphthoflavone (BNF) (40 mg/kg) daily for three days as described in Yilmazer *et al.*, 2001a. BNF, formic acid, uridine 5'-diphospho-glucuronic acid (UDPGA), and β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile, ethanol, and methanol were HPLC grade from Mallinckrodt Baker, Inc. (Paris, KY).
Xanthohumol Biotransformation or Glucuronidation by Rat Liver Microsomes

A typical 85 ml biotransformation incubation mixture consisted of 85 mg rat liver microsomal protein, a NADPH generating system with glucose-6-phosphate containing 0.25 mM MgCl₂ and 100 μM XN (dissolved in 170 μl ethanol) as substrate (Yilmazer et al., 2001a). The reaction was initiated by adding 1 mM NADPH. Incubation was carried out at 37°C for 60 min with continuous shaking in a Dubnoff incubator. Reactions were terminated by adding 17 ml ice-cold methanol, followed by centrifugation at 4°C.

A typical 36 ml glucuronidation incubation mixture consisted of 36 mg rat liver microsomal protein, 52 mM HEPES/NaOH buffer-pH 7.4 containing 10 mM MgCl₂, 0.25 mM triton X-100, 52 mM UDPGA, and 100 μM XN (dissolved in 216 μl ethanol) as substrate (Yilmazer et al., 2001b). The reaction was initiated by adding UDPGA. Incubations were carried out at 37°C for 60 min with continuous shaking in a Dubnoff incubator. Reactions were terminated by adding 1.5 ml ice-cold methanol, followed by centrifugation at 4°C. The supernatants from either of the incubations were lyophilized after evaporation of methanol by a rotary evaporation under vacuum. The residue was redisolved in 25% CH₃CN containing 1% formic acid and run in preparative HPLC.

Isolation of Metabolites and Glucuronides by Preparative-HPLC

The redisolved metabolites or glucuronides were isolated by preparative HPLC with an Alltech Econosil C₁₈ (250 x 22 mm) column at a flow rate of 11 ml/min by using acetonitrile and 1% of formic acid in H₂O. The initial 29% CH₃CN was increased to 60% over 18 min and then to 84% over the next 10 min with a linear gradient function. At 34 min, the CH₃CN was returned to 29% in 4 min. Fractions were collected according to their retention time at 370 nm absorption. Methanol was evaporated from collected fractions and samples were lyophilized overnight.
Two oxidation products from the NADPH-mediated reaction, M2 (5"-(2-hydroxyisopropyl)-dihydrofurano[2",3":3',4"]-2',4-dihydroxy-6'-methoxychalcone) and M4 (6", 6"-dimethylpyrano-[2",3":3',4"]-2',4-dihydroxy-6'-methoxychalcone), and two glucuronides formed in the presence of UDPGA, G1 (OH-4' glucuronide) and G2 (OH-4 glucuronide) were run in a Waters 2690 HPLC system with a 996 diode-array detector and a 4 µm Nova-Pak C18 column (3.9x150 mm, Waters) to check the purity of the metabolites before using them in the antioxidant assays.

**Assessment of AAPH Catalyzed LDL Oxidation**

The metabolites; M4 or M2 (0.5 or 5 µM, in ethanol), and glucuronides; G2 or G1 (1 or 5 µM, in ethanol) and XN (from 1 to 5 µM, in ethanol) were added in 1 µl volume to LDL in a total volume of 200 µl/well of a 96-well plate. The final concentrations of the NADPH mediated metabolites, M2 and M4, and UDPGA mediated glucuronides, G1 and G2, were not uniform because of the difficulties in preparing the stock solutions from their very limited amounts.

Immediately prior to the lipid peroxidation assay, LDL was diluted in PBS to a concentration of 0.2 mg/ml LDL (final concentration of 0.1 mg/ml) was incubated at 37°C with the individual XN metabolites or glucuronides and AAPH (5 mM) in phosphate buffered saline, pH 7.4, in a total volume of 200 µl. The tubes were incubated with slow shaking in a water bath set at 37°C for 4 h before the amount of TBARS were measured as described by Buege and Aust (1978) with some modifications. Twenty µl of ice-cold 50% trichloroacetic acid was added into each tube and vortexted before adding 100 µl of 1% thiobarbituric acid in 0.28% NaOH to each tube. Then, the samples were acidified by the addition of 20 µl of 1 N HCl. The acidified samples were heated at 90°C for 20 min and centrifuged at 16,000 x g for 5 min. An aliquot (200 µl) of the supernatant was transferred to a 96-well plate and the absorbance at 535 nm was measured using a microplate reader (SpectraMax 250).
Ethanol (1 μl/well) was added to LDL in control wells instead of XN. Other sets of experiments, containing PBS (no LDL) or AAPH alone, were carried out to make an estimate of any interference of AAPH or PBS in the TBARS assay.

**Results and Discussion**

The proposed binding sites for trace metals to flavonoids are the catechol moiety in ring B, the 3-hydroxyl, 4-oxo groups in the heterocyclic ring, and the 4-oxo, 5-hydroxyl groups between the heterocyclic and the A rings (Pietta 2000; Rice-Evans and Miller, 1996). Although XN does not have the catechol moiety in ring B or a 3-hydroxyl group, it has been reported earlier that XN inhibits copper-mediated oxidation of LDL (Miranda *et al.*, 2000). Also it has been suggested that the presence of a methoxy group at the 6' position in XN increases the antioxidant activity against AAPH-induced oxidation of LDL (Stevens *et al.*, 2001).

Recently, it has been shown that flavonoids might be metabolized *in vivo* yielding metabolites that exhibit antioxidant properties (Morand *et al.*, 1998). In the present study, two oxidation products from the NADPH-mediated reaction, M2 (5"-(2-hydroxyisopropyl)-dihydrofurano[2",3":3',4']-2',4-dihydroxy-6'-methoxychalcone) and M4 (6", 6"-dimethylpyrano-[2",3":3',4']-2',4-dihydoxy-6'-methoxychalcone), and two glucuronides formed in the presence of UDPGA, G1 (OH-4' glucuronide) and G2 (OH-4 glucuronide) (Figure 6.1) were analyzed with respect to their effects on TBARS formation from LDL and compared to each other and to known antioxidant concentrations of XN. All calculations were done considering ethanol effects on LDL as control because all substrates were dissolved in ethanol.
Figure 6.1. The chemical structures of XN, M2, M4, G1, G2.
Figure 6.2 shows the inhibitory effects of XN and its oxidation metabolites and glucuronides on AAPH-mediated lipid peroxidation in LDL. At 5 μM M2 showed comparable antioxidant activities to that seen with 5 μM XN as shown by the inhibition of TBARS formation. Also, at 0.5 μM M4, the nonpolar metabolite of XN, showed comparable antioxidant activities to that of seen with 1 μM XN. However, glucuronides, G1 and G2, at 5 μM and 1 μM, respectively, enhanced TBARS suggesting a prooxidant activity.

Figure 6.2. The effects of XN, M2, M4, G1, and G2 on lipid peroxidation (measured as TBARS) induced by AAPH in human low-density lipoproteins.
The M2 metabolite of XN, formed by rat liver microsomes, was identified as 5''-(2'''-hydroxyisopropyl)-dihydrofurano[2''',3''':3',4']-2',4-dihydroxy-6'-methoxychalcone by NMR in our previous study (Yilmazer et al., 2001a). This study showed that the attachment of a furan ring to the A ring of XN still retained the antioxidant property of XN flavonoid.

The nonpolar metabolite of XN, M4 (6'', 6''-dimethylpyrano-[2''',3''':3',4']-2',4-dihydroxy-6-methoxychalcone or dehydrocycloxanthohumol), formed by rat liver microsomes (Yilmazer et al., 2001a) is a flavonoid present in hops (Stevens et al., 1997). The present study showed that the nonpolar in vitro metabolite of XN showed antioxidant activity against AAPH-induced LDL oxidation. This agreed with the previous study that utilized dehydrocycloxanthohumol isolated from hops and showed its antioxidant activity in the same system (Stevens et al., 2001).

The results of the present study indicted that XN glucuronides were not capable of inhibiting AAPH induced oxidation of LDL, but rather exerted prooxidant effects on LDL oxidation, based on TBARS formation. The absence of antioxidant activity for both the 4'- and 4-glucuronides of XN suggested that both hydroxyl groups were involved in the protective effect of XN against lipid peroxidation. The absence of an antioxidant activity of G1 and G2 was contradictory to studies performed with quercetin glucuronides obtained via an in vitro procedure which showed antioxidant properties toward rat VLDL+LDL fractions during oxidation with CuSO₄ (Morand et al., 1998). On the other hand, in the presence of a peroxidase, flavonoid compounds with B rings containing phenolic groups might form prooxidant phenoxy radicals, which during redox cycling, could generate superoxide radicals and hydrogen peroxide as suggested for naringenin (Sahu and Gray, 1997). The glucuronidation in the B ring of XN also caused the generation of superoxide radicals and hydrogen peroxide and showed prooxidant properties.

Our data also showed that the absence of the prenyl group in the metabolites did not result in the disappearance of the antioxidant activity against LDL oxidation induced by AAPH. Although it was suggested that the increased number of prenyl
group might result in a loss of antioxidant activity (Stevens et al., 2001), it is possible that the prenyl group is not necessary for antioxidant activity.

Studies on structure-activity relationships have shown that the presence of both hydroxyl and methoxy groups in the A and B rings appear to be important in antioxidant and free radical scavenging activities of chalcone compounds (Anto et al., 1995). The antioxidant properties of the hydroxylated metabolites of XN formed by human liver microsomes or cDNA-expressed human cytochrome P450s (Yilmazer et al., 2001c) remains to be established.

Based on the formation of TBARS, the antioxidant activity of the beverages was ranked as follows: black tea > coffee > prune juice = beer > green tea > orange juice > red wine > tangerine juice > red grape juice > white grape juice > grapefruit juice (Vinson et al., 1999). The antioxidant activity of the fruit juices and tea could be explained by their high content of polyphenolic flavonoids. However, the specific compounds responsible for the antioxidant activity in beer or the activity of their metabolites have not been identified. The potential impact of XN found in beer and hops, with respect to the antioxidant properties of XN and its metabolites, on human health is not known. It needs to be investigated whether XN is absorbed and the metabolites have the same antioxidant activity in vivo.

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References


CHAPTER 7

APPROACH TO RISK ASSESSMENT EVALUATION OF HUMAN HEALTH RISKS OF FLAVONOIDS AND XANTHOHUMOL FROM FOOD

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Abstract

Human health risks from flavonoids, and specifically from prenylated chalcone flavonoids present in beer and dietary supplements, were evaluated by considering findings related to flavonoid toxicity in general.

The daily intake of xanthohumol (XN) and total prenylated flavonoids (TPF) is estimated at 0.007 and 0.014 mg/kg/day, respectively, for moderate beer drinkers (assuming two 12 ounce beers per day). As the beer companies may want to increase the amount of XN in beer, the risk of this increase was evaluated. The risk from a dietary supplement of capsules containing 1 mg/kg XN was also evaluated.

Results of this evaluation suggest that flavonoids detected in hops and the amount of XN in beer are not likely to cause any immediate toxicological problems for humans. Because of a lack of experimental data, further toxicological evaluations are necessary to quantitatively evaluate the risk.

Introduction

The risk for humans from food is a function of the probability and severity of an adverse effect. Risk can be predicted from determination of the hazard and human exposure to this hazard. The human need for food is obvious, and food will present some risk because all potential hazards cannot be eliminated. Therefore, there is a clear need to be able to assess the risk of food consumption in order to protect the consumer (1).

For human health assessment, risks are evaluated by aggregating data from predefined data groups and estimating risks from the aggregated concentrations. However, the risk assessment for foods is complicated as biological systems are involved. For this reason, it is usual to use conservative models, add in large safety factors and then still equivocate on whether a particular intake of a food ingredient is absolutely safe (1).
In this study we evaluated the risks to humans from flavonoids, ubiquitous phenolic compounds in foods, and specifically from prenylated chalcone flavonoid in hop (*Humulus lupulus*) flower extracts (2) and in beer (3).

**Risk Assessment Approach**

Risk is a relative word since the risk may be real or it may be perceived as such by society (1). There is not a single, fixed method of risk analysis, rather, it is a systemic approach to organizing and analyzing scientific knowledge and information for potentially hazardous activities or for substances that might pose risks under specified conditions (4).

According to United States Environmental Protection Agency (5), risk assessment can be divided into three steps: toxicity assessment, exposure assessment, and risk characterization (4,5).

Toxicity Assessment has two parts. The first is hazard identification that is the process of determining what adverse health effects (e.g., cancer, estrogenicity) can result from excessive exposure to a particular chemical and whether the adverse health effects could occur in humans. It involves characterizing the nature of the link between exposure to a given chemical and a biological effect(s) (5). The second part is dose-response assessment that characterizes the relationship between exposure or dose and the incidence and severity of the adverse health effect. It includes consideration of factors that influence dose-response relationship, such as intensity and pattern of exposure and age and lifestyle variables that could affect susceptibility. It can also involve extrapolation of high-dose responses to low-dose responses and from animal responses to human response.

Exposure Assessment is the determination of the intensity, frequency, and duration of actual or hypothetical exposures of humans to the agent in question. Modeling is often used to establish the relationship between emissions and environmental concentrations of the substance.
Risk characterization combines the assessments of exposure and response under various exposure conditions to estimate the probability of specific harm to an exposed individual or population.

Risk management is closely related to risk assessment. It integrates the result of risk assessment with other information—such as political, social, economic, and engineering considerations—to arrive at decisions about the need and methods for risk reduction. Risk communication represents evaluations to the public (4).

**Toxicity Assessment of Flavonoids and Xanthohumol**

The purpose of the toxicity assessment is to weigh the available evidence regarding the potential for particular compounds to cause adverse effects in exposed individuals and to provide, where possible, an estimate of the relationship between the extent of exposure to a contaminant and the increased likelihood or severity of adverse effects (5).

USEPA has performed the toxicity assessment for many chemicals and has published the resulting toxicity information and toxicity values on the IRIS and HEAST databases (5).

Overall, the dietary studies have shown positive effects of flavonoids that may be interpreted as beneficial, but it is difficult to identify the precise contribution that flavonoids play in the overall endpoint measures in humans.

**Hazard Identification**

Flavonoids are the most common and widely distributed group of phenolics in plants. More than 5000 chemically unique flavonoids have been identified in plant sources by 1990. As they are ubiquitous and found in fruits, vegetables, nuts, seeds, stems, flowers as well as tea and wine, they are an integral part of the human diet (6,7).

Interest in the physiologic role of the bioactive compounds present in plants has increased dramatically over last decade. Recently, it has been hypothesized
that the antioxidant properties of flavonoids may protect tissues against oxygen free radicals and lipid peroxidation, both of which may be involved in several pathological conditions such as atherosclerosis, cancer and chronic inflammation (8). The anticancer activity of some flavonoids also has been studied and their growth-inhibitory activity has been shown in human breast cancer cells, colon cancer cells lines, a lymphoblastoid cell line, acute lymphoid and myeloid leukemia cell lines, and squamous cell carcinoma cell lines (9).

In addition to in vitro and in vivo studies, epidemiological studies also suggest that consumption of flavonoids from fruit and vegetables may have protective effect against some diseases. The intake of flavonoids is inversely associated with the risk of coronary heart disease in elderly men and protects against stroke (10). The epidemiologic data also indicates that Asian women consuming a diet high in soy are less susceptible to mammary cancer (11).

For assessing human risks, adverse health effects are divided into two broad groups: noncancer effects and cancer. There is no evidence of carcinogenicity in the flavonoid studies that have been done. Noncancer health effects include a variety of toxic effects on body systems ranging from renal toxicity to hormonal disorders. Noncancer health effects fall into two basic categories: acute and chronic toxicity. Apparently there is no acute toxicity of flavonoids as they have been a part of the diet for years. However, there are some concerns about their adverse effects in chronic toxicity, such as flavonoid-drug interactions or estrogenic effects.

The mutagenicity of flavonoids in bacterial systems has triggered much research (8,12). There is the potential for some chemicals, including flavonoids, to be converted to electrophilic metabolites by cytochrome P450s (CYPs) (13). Furthermore, MacGregor and Jurd (14) reported an increase in the mutagenic activity of kaempferol, a flavonoid in apple and red wine, subsequent to treatment with rat liver microsomes. They hypothesized that the increased mutagenicity occurred by microsomal hydroxylation of the B ring in kaempferol, resulting in the formation of quercetin, a flavonoid in tea and vegetables. Also redox cycling
between catechols, flavonoids found in tea, and the corresponding quinones has been suggested as the primary event responsible for the observed mutagenicity of quercetin in vitro (15).

Dietary administration of flavonoids to rats is reported to cause significant increases in hepatic CYP catalyzed reactions such as ethoxyresorufin, pentoxyresorufin and ethoxycoumarin dealkylases (16). Flavonoids reportedly have striking effects on the CYP-dependent monooxygenase system, including the induced synthesis and activation or inhibition of specific CYP isozymes (17,18). The effects of flavonoids on CYPs are complex and may result in pharmacological and toxicological alterations in vivo (18).

The CYP isozyme primarily involved in the metabolism of flavonoids is CYP1A1, but CYP2B and CYP3A forms might also be involved (15). CYP1, CYP2 and CYP3 families have a major role in the detoxification of foreign chemicals (19). CYP1A1 is among the best studied of the CYPs due to the conservation of its catalytic activities and regulation among different species. CYP1A1 is involved in the metabolic activation of polycyclic aromatic hydrocarbon procarcinogens. The fact that it is inducible by other chemicals, however, raises a concern that a drug or other chemical may induce an enzyme that is capable of activating procarcinogens (13).

Recently, considerable attention has been paid to the ability of flavonoids to interact with drug-metabolizing enzymes that are involved in mammalian metabolism of various lipophilic xenobiotics, such as drugs, environmental pollutants and insecticides (15). It has been established that grapefruit juice increases oral bioavailability of nifedipine, felodipine, nitrendipine, cyclosporine, nisoldipine, nimodipine, verapamil, and terfenadine, prolongs caffeine half life, and delays urinary excretion of 7-hydroxycoumarin after coumarin administration. The mechanism of this grapefruit juice-drug interaction is understood only in part, but naringenin, the metabolite of the major grapefruit juice flavonoid, naringin, is found to be responsible for the interaction. Naringenin is a potent inhibitor of
CYP1A2 and CYP3A, isoforms that are important for the detoxification of many drugs (20).

Some studies conducted in humans clearly confirm that one class of flavonoids, the isoflavones, are responsible for altered hormonal activities in animals and *in vitro*. This suggests a plausible mechanism for physiological changes in human due to diets rich in these compounds. Consequently, the presence of high concentrations of isoflavonoids in soy-based infant formula has been a controversial issue (21).

More specifically, hop plants have been used for the treatment of gynecologic disorders and, in earlier days when hops were picked by hand, menstrual disturbances among female pickers reportedly were common. The estrogenic activity of hops was initially attributed to XN, the principal flavonoid of hops (2), but without any evidence supporting that assertion (22). However, further investigation has shown that another flavonoid in hops, 8-prenylnaringenin, is responsible for this estrogenic effect (23). As hops are added into beer, it is thought that beer may also be estrogenic (22,23).

Also hop-containing brewery dust extracts have been shown in guinea pigs to cause a dose-related airway smooth muscle constriction by nonimmunological mechanisms involving cholinergic receptors and a variety of airway mediators (24). Bronchial irritation and chronic bronchitis has been reported in hop packing and storing workers (25), and brewery workers have been noted as having a higher prevalence of positive skin prick test and chronic respiratory symptoms (26). It was also reported that, secondary to the ingestion of spent hops, dogs suffered adverse effects including hyperthermia, restlessness, excessive panting, vomiting, signs of abdominal pain, and seizures. Four of the five dogs died despite aggressive therapeutic measures (27).

CYPs involved in XN metabolism are CYP1A1, CYP1A2, and CYP3A4 (28). As CYP3A4 inhibition with naringenin from grapefruit juice has been reported, this type of interaction with other flavonids and XN also needs to be investigated.
Dose-Response Evaluation

The development of toxicity is dependent on several factors including, the dose of a chemical, the route of administration or exposure, and individual susceptibility. The dose-response relationship is a quantitative expression of the response observed in a test population (either humans or animals) at a given dose or range of doses. These values are expressed as noncancer reference doses (RfD) and cancer slope factors (SF), both of which are exposure route-specific.

The primary source of critical toxicity values is USEPA’s Integrated Risk Information System (IRIS) data base. The secondary source of critical toxicity values is the Health Effects Assessment Summary Tables (HEAST). Neither of these have RfD values for any of the flavonoids. It should be recognized, however, that the lack of an available toxicity value does not preclude a chemical’s toxicity (5).

In general, the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to occur without an appreciable risk of deleterious effects during a life time. The RfD is determined by use of the following equation and is generally expressed in units of milligram per kilogram of body weight per day (mg/kg/day):

\[ \text{RfD} = \frac{\text{NOAEL or LOAEL}}{\text{UF} \times \text{MF}} \]

NOAEL is the exposure level at which there are no statistically significant increases in the frequency or severity of adverse effects between the exposed population and its appropriate control. LOAEL is the lowest exposure level at which there are statistically or biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control group. Uncertainty factor (UF) is one of several, generally tenfold, factors used in operationally deriving the RfD from experimental data.
Modifying factor (MF) is an uncertainty factor that is greater than zero and less than or equal to 10; the default value for MF is 1.

It was reported that the LD_{50} values of preisomerized and reduced hop extracts were approximately 1 g/kg body weight in the rat when these were administered as single doses in a 50% solution in corn oil. These extracts were also fed at levels of 1, 0.1, and 0.01% in the diet to Sprague-Dawley rats for 90 days. A reduction in body weight gain was observed at the 1% dietary level for both extracts but no changes were observed in the weights of the liver, heart, spleen, kidneys or gonads, compared with controls and no histopathological changes related to treatment were noted in rats (29). However, the data was insufficient to determine LOAEL from this study.

In one study, a NOAEL was determined as 25 mg/kg for XN in 6 female CD-1 mice after oral administration for 4 consecutive days with XN (30). According to this data, RfD was calculated as;

\[
RfD = \frac{25 \text{ mg/kg}}{(10 \times 10 \times 10)} \times 3 = 0.0083 \text{ mg/kg/day}
\]

A tenfold factor was used to account for the uncertainty in extrapolating animal data circumstances to human exposure for XN. Yet, another tenfold factor was added for variation in sensitivity among humans. As the database did not contain results from chronic studies in at least two species nor reproductive or developmental results, a third tenfold factor was used to account for this uncertainty. The default value, 3, was used as modifying factor (4).

**Exposure Assessment of Flavonoids and Xanthohumol**

Exposure is defined as the contact of an organism with a chemical or physical agent. Exposure assessment is the determination or estimation (qualitative or quantitative) of the magnitude, frequency, duration, and route of exposure. The magnitude of exposure is determined by measuring or estimating the amount of an
agent available at the exchange boundaries (i.e., the lungs, gastrointestinal tract, and skin) (5).

Exposure to foreign chemicals, including food, can be examined by a variety of methods such as direct determination of levels of the substance and its metabolites in urine or by analysis of protein and DNA-bound metabolites in body fluids. The reliability and accuracy of these measurements are, in large part, dependent on the extent of exposure, the chemical’s half-life in the body, and the sensitivity of the detection method (13).

The main intake route of flavonoids is oral administration. Although they are almost ubiquitous in plant foods (vegetables, cereals, legumens, fruits, nuts, etc.) and beverages (wine, cider, beer, tea, cocoa, etc.), the daily consumption of flavonoids is extremely variable according to dietary habits (6). In the United States the intake of dietary flavonoids is estimated to be between 1 and 1.1 g/day, depending on the season. However, the improvement of the analytical techniques suggests 3-70 mg is a more accurate estimation (31).

More specifically, most soy products contain quite large amounts of genistein (1-3 mg/kg), an isoflavonoid, and daidzein, a flavonoid in vegetables (32). In another study, the amount of genistein is reported as 1g/kg in soy beans (33). It is estimated that quercetin, a major flavonol in fruit and vegetables, is responsible for 60-75% of the total flavonoid exposure in humans from diet (31).

USFDA has reported hops as ingredients in the following six products; K8, Hi-Stress B&C, Eight Combination, Natural Sterol Complex, Somnist Melatonin Complex, and Nature’s Way Stress. They are used for treatment of anxiety, stress, insomnia, severe chest pain, etc... (34). A novel dietary supplement composition that has Kava root and 50 mg hops was patented in June, 2000 in a capsule form and recommended as a general relaxant (35). A method was also patented for treatment of skin with a hop extract to protect the skin from erythema-producing sunlight radiation while promoting tanning (36). This method involves using an active sunscreen ingredient, an ultraviolet radiation absorbing extract of hops.
The brewing industry uses female inflorescences (hop cones or 'hops') to give beer its characteristic flavor and aroma. This makes beer the major dietary source of prenylated flavonoids (2). The total prenylated flavonoid content of beer is estimated as high as 4 mg/L, or approximately 11 μM (based in the molecular weight of XN) (3,37).

XN, the principal prenylated flavonoid present in hops (2) is reported at concentrations as high as 0.69 mg/L (1.95 μM) in beer (3). XN constitutes 82-89% of the total amount of prenylated flavonoids of different European hop varieties (2). XN is patented for use as an inhibitor of bone resorption (38). It also exhibits high antifungal activity against Trichophyton spp. (39,40).

**Estimation of Intake**

The quantification of exposure is based on an estimate of the chronic daily intake (CDI) which is expressed in terms of milligrams of chemical contacting the body per kilogram body weight per day (mg/kg/day) (5). The following generic equation is used to estimate the CDI:

\[
CDI \text{ (mg/kg body weight/day)} = \frac{C \times IR \times EF \times ED}{BW \times AT}
\]

C= Concentration of chemical (e.g., mg/kg or mg/L)
IR= Intake rate (e.g., kg/day or L/day)
EF= Exposure frequency (days/year)
ED= Exposure duration (years)
BW= Body weight (kg)
AT= Averaging Time (period over which exposure is averaged, days)

The exposure parameters used in the equation are specific to a particular exposure scenario. Exposure values are often assumed values and their magnitude influences the estimates of potential exposure (risk) (5).
In this report, we evaluated the exposure of total prenyalted flavonoids and XN from beer and dietary supplements. The amount of XN and TPF differs according to beer type and brewing processess.

**A) Concentrations based on the beer research:**

It has been shown that the maximum amount of XN in beer is 0.69 mg XN/L or 4.0 mg/L TPF, respectively (3).

**B) Concentrations based on an assumption:**

As discussed in chapter 6, antioxidant properties of XN have been demonstrated at 1 µM concentrations in *in vitro* studies against human LDL oxidation induced by AAPH (41). If we assume that a 1 µM XN plasma concentration will have antioxidant properties in humans, we can calculate the amount of XN in a capsule needed to achieve this effect in humans. However, no data about XN absorption and distribution in humans was available, therefore, we assumed that XN had 100% bioavailability in the body and would be restricted to plasma due to its polar character and did not enter other tissues (42). A 70 kg human has 3 liters plasma water, therefore, the amount of XN needed for the antioxidant activity would be 1 mg in a 70 kg human (1 µM XN = 354 µg XN/L).

According to this scenario, a person can take one XN capsule containing 1 mg XN as a dietary supplement or drink 1 L beer that has 1 mg/L XN per day to reach this concentration in plasma.

For CDI calculations, other values besides these concentrations discussed above were as follows:
EF: 365 days/year
ED: 30 years
BW: 70 kg
AT: 10950 days (365 days x 30 years)

CDI of XN is calculated as 0.007 mg/kg/day for a moderate alcohol drinker (two 12 ounce beers /day) by drinking a beer with 0.69 mg/L XN concentration. Assuming a 70 kg individual takes a capsule that has 1 mg XN/capsule or consumes a liter of beer with 1 mg/L XN, CDI would be 0.014 mg/kg/day (Table 7.1).

Table 7.1 Exposure Assessment

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>IR</th>
<th>CDI (mg/kg/day)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>XN in beer</td>
<td>0.69 mg/L</td>
<td>2 beers/day</td>
<td>0.007</td>
<td>Stevens et al., 1999</td>
</tr>
<tr>
<td>XN in a capsule</td>
<td>1 mg/capsule</td>
<td>1 capsule/day</td>
<td>0.014</td>
<td>Assumption</td>
</tr>
<tr>
<td>XN in beer</td>
<td>1 mg/L</td>
<td>1 L/day</td>
<td>0.014</td>
<td>Assumption</td>
</tr>
<tr>
<td>TPF in beer</td>
<td>4 mg/L</td>
<td>2 beers/day</td>
<td>0.040</td>
<td>Stevens et al., 1999</td>
</tr>
</tbody>
</table>

Human Health Risk Characterization

This section summarizes the approach used to develop the human risk estimates for chemicals and presents a quantitative risk characterization. This process is different for carcinogens and noncarcinogens. As flavonoids are considered noncarcinogenic compounds, their hazard quotient is calculated by the equation below:

Hazard Quotient (HQ) = CDI / RfD
If the HQ is less than 1, the chemical exposure under consideration is regarded as unlikely to lead to adverse health effects. If the HQ is greater than 1, more detailed evaluations is needed. Also, if RfD is based on a serious health effects in humans, HQ will raise more concern than that based on animal data.

A) Based on a beer that has 0.69 mg XN/L (3):

HQ is calculated for XN as \( \frac{0.007}{0.0083} = 0.8 \) for a moderate beer drinker (two 12 ounce beers /day).

As the HQ for XN does not exceed 1, the chemical exposure under 0.007 mg/kg/day is regarded as unlikely to lead to adverse noncancer effects.

B) Based on that has 1 mg XN/L or a dietary supplement that has 1 mg XN/capsule:

For someone who takes a capsule that has 1 mg XN/day or drinks 1 L of beer having 1 mg XN/L, HQ is calculated as \( \frac{0.014}{0.0083} = 1.7 \).

In this case, HQ for XN exceeds 1. However, it is important to consider that the RfD used to calculate the HQ is based on only one study and includes a number of uncertainty factors. Also, the assumption to find CDI is based on the results of the \emph{in vitro} antioxidant experiments with XN. The HQ is simply an estimate of risk based on previous study data. The actual risk may be much lower and could even be zero.

**Risk Assessment Uncertainties**

Uncertainties associated with sampling and analyses include the inherent variability (standard error) in the analysis. The efficiency of the analytical methods, representativeness of the samples, sampling errors, and heterogeneity of the sample matrix may vary in quantification of flavonoids. The inclusion of these analytical variables in risk estimate results in an overestimate of risk.
On the other hand, the consumption rate of flavonoids from food is difficult to estimate as the amount of flavonoids vary in food by season, species, and storage. However, flavonoids are resistant to boiling and fermentation (43), and cooking method will not effect the amount of them in food. Dietary habits have an important role in the intake of flavonoids, however, the extent of absorption, bioavailability and metabolism of flavonoids are not well known in humans. In this study we assume 100% bioavailability of XN. The estimates in risk assessment are based in the intake of flavonoids which is likely different than the absorbed amount of flavonoids. Also, the biological activities of flavonoid metabolites remain to be established. Depending on whether flavonoid metabolites are more or less toxic than the parent compound, these assumptions could result in an under- or over-estimation of exposure and risk. XN may have beneficial effects which could change the magnitude of the risk, however it remains to be established. The synergistic, additive effects of other flavonoids with XN and interactions between ethanol and XN remain to be established.

Calculations depend on the assumption that the concentration of flavonoids in diet or specifically, XN in hops or beer, remain constant over the duration of exposure. However, the amount of flavonoid intake changes according to the dietary habits of humans and the levels of flavonoids which vary greatly between cultivars of the same species. Also storage conditions may cause the oxidation of flavonoids (6). As the pharmacokinetics of XN was not completely understood, for the dietary supplement scenario we assumed steady state condition in serum and no metabolism.

The RfD used in this risk assessment is a major source of uncertainty. It was based on only one study and included a number of uncertainty factors. Therefore, the confidence rating for RfD was low. There were no other studies available from which an RfD for xanthohumol could be calculated.
Summary

The noncancer risk estimated from oral exposure to XN for beer consumption is below a level of concern. Although a capsule with 1 mg XN or 1 L of beer with 1 mg/L concentration showed a HQ slightly higher than 1, we believe this is represents an extreme exposure that has a low likelihood of occurrence. Therefore, it is unlikely that consumption of 1 mg XN via beer or a capsule will result in toxicity due to XN. It is not known if flavonoids from other dietary sources may have synergistic or additive effects with XN.

Studies conducted in humans indicate that flavonoids may have adverse effects such as estrogenic effects or flavonoid-drug interaction. Since biological effects are dependent on many factors, including dose, duration of use, protein binding affinity, individual metabolism, and intrinsic factors, further toxicological, clinical and epidemiological studies are necessary to determine the potential health effects of flavonoids, including XN, in specific populations.
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SUMMARY AND CONCLUSIONS

In vitro and in vivo metabolism of xanthohumol (XN), a flavonoid in hops and beer, has been studied in rats and human liver microsomes. XN was metabolized to the three metabolites, M1, M2, and M3 by rat liver microsomes. Liver microsomes from rats pretreated with the CYP1A inducers, ISF and BNF (Lewis, 1996), yielded the three same polar metabolites plus an additional nonpolar metabolite, designated M4. The formation of these metabolites required NADPH.

On the basis of multi wavelength HPLC, LC/MS analysis and \(^1\)H-NMR analysis, M1 and M2 were both identified as hydroxylated isopropylidihydrofurano derivatives of XN. LC/MS analysis of the liver microsome incubations allowed M3 to be characterized as either 2- or 3-hydroxyxanthohumol. M4 was identified as the dimethylchromeno analog, DX. These results indicate that the prenyl group in the A ring of prenylchalcones was the a major site for hepatic metabolism. The formation of metabolites such as M2 and M4 suggested that modification of the prenyl substituent, involving an intermediate epoxidation of the double bond, constitutes a major metabolic pathway of prenylated flavonoids, although it has been reported that catechol formation on the B-ring of non-prenylated flavonoids was a major site for CYP hydroxylation (Nielsen et al., 1998).

The amount of XN metabolites produced by rat liver microsomes was influenced by the use of various CYP inducers. Therefore, the role of individual
CYPs in the metabolism of XN was investigated by using cDNA expressed human liver CYPs. XN was a selective substrate of human CYP3A4, CYP1A1, and CYP1A2 enzymes in the metabolism of XN. Specifically, M1, M2, and M3 formations were mediated by CYP3A4. The nonpolar metabolite of XN, M4, was produced by c-DNA expressed human CYP1A1 or CYP1A2. Also human liver microsomes formed M1, M2, M3, and M4. Overall, the results suggested that the major pathways for the metabolism of XN by cDNA expressed human CYPs and human liver microsomes were the formation of the hydroxylated XN or the cyclization of XN.

XN glucuronidation also was investigated by rat and human liver microsomes. The chemical structures of two major glucuronides, G1 and G2, of XN were identified by HPLC, LC/MS analysis and ¹H-NMR. Glucuronidation of XN at position C4' (G1) appeared to be favored above glucuronidation at C4 (G2) in these experiments. This suggested that the presence of a prenyl group in the A ring did not preclude glucuronyltransferase catalyzed O-glucuronidation of XN as expected due to limited acceptability of the substrate as a result of steric hindrance.

The metabolism studies with cDNA expressed human CYPs and human liver microsomes indicated that XN was a selective substrate of human CYP3A4, CYP1A1, and CYP1A2 enzymes. The results suggested that the major pathways for the metabolism of XN by cDNA expressed human CYPs and human liver microsomes were the formation of the hydroxylated XN or the cyclization of XN.

*In vivo* studies on the metabolism of XN showed that it was absorbed and formed glucuronides quickly, this extensive conjugate formation led to its rapid excretion in urine. Interestingly, demethylation was not observed during the metabolism of XN by rat or human liver microsomal enzymes or cDNA expressed human liver microsomes in contrast to other flavonoids with O-methyl groups. The lack of a C ring or the presence of a prenyl group in the A ring might preclude CYP catalyzed O-demethylation of XN, possibly due to limited acceptability of the substrate as a result of steric hindrance. The fact that plasma contained mainly glucuronide conjugates demonstrated effective conjugation of XN.
It has been shown that flavonoids are conjugated in vivo, yielding flavonoid glucuronides that exhibit a variety of biological activities, such as antioxidant. Previous studies have shown that XN is an effective inhibitor of AAPH-induced oxidation of LDL. In present study the inhibitory effects of XN oxidation metabolites and glucuronides on lipid peroxidation in LDL exposed to AAPH were studied by comparing that of the parent XN. At 5 μM M2 showed comparable antioxidant activities to that of seen with 5 μM XN as shown by the inhibition of TBARS formation. Also, at 0.5 μM M4, the nonpolar metabolite of XN, showed comparable antioxidant activities to that of seen with 1 μM XN. However, glucuronides, G1 and G2, at 5 μM and 1 μM, respectively enhanced TBARS suggesting a prooxidant activity.

Human exposure to XN, the principal flavonoid of hops, would be primarily through the drinking of beer or ingestion of dietary supplements containing hop extracts or of herbal drugs. Risk evaluation studies suggest that XN is not likely to cause chronic toxicity for humans either from beer or from dietary supplements.

Further studies are needed about;

- The bioavailability of XN in humans
- XN metabolism in humans
- The biological effects of metabolites and their involvement in cancer chemoprevention
- Dose-response studies of XN for risk evaluation

In conclusion, this work has demonstrated that XN is metabolized by rat and human liver microsomes. CYP3A4, CYP1A1, and CYP1A2 are the major responsible isozymes. XN is absorbed and formed glucuronides quickly in rats, this extensive conjugate formation leads to its rapid excretion in urine. XN does not appear to undergo any other significant metabolic transformation in rats. Two of the oxidized metabolites of XN have promising antioxidant properties. However,
two glucuronides of XN have prooxidant activity by inducing oxidation of LDL induced by AAPH. There is no available data about XN toxicity.
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