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IDENTIFICATION OF HUMAN HEPATIC CYTOCHROME P450 ENZYMES INVOLVED IN THE METABOLISM OF 8-PRENYLNARINGENIN AND ISOXANTHOHUMOL FROM HOPS (*HUMULUS LUPULUS* L.)

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Abbreviations

HPLC: high performance liquid chromatography-mass spectrometry

HLM: human liver microsomes

IX: isoxanthohumol

IX-M1: isoxanthohumol *cis* prenyl alcohol

IX-M2: isoxanthohumol *trans* prenyl alcohol

LC-MS: liquid chromatography-mass spectrometry

8PN: 8-prenylnaringenin

8PN-M1: 8-prenylnaringenin *cis* prenyl alcohol

8PN-M2: 8-prenylnaringenin *trans* prenyl alcohol

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Abstract

The female flowers of hops (*Humulus lupulus* L.) are used in the brewing of beer and are under investigation for use in dietary supplements for the management of menopausal symptoms in women. Hop extracts contain the weakly estrogenic compound isoxanthohumol, pro-estrogenic xanthohumol, and the potent estrogen 8-prenylnaringenin. Since isoxanthohumol can be metabolized in the human liver to form 8-prenylnaringenin, the specific cytochrome P450 enzymes responsible for this *O*-demethylation reaction were identified. In addition, the enzymes that convert isoxanthohumol and 8-prenylnarigenin to their most abundant metabolites were identified, since these metabolic pathways might also affect the estrogenicity of hop preparations. Specifically, the CYP450 enzymes that catalyze the oxidation of the prenyl side chains of isoxanthohumol and 8-prenylnarigenin into *trans* or *cis* alcohols were investigated. Human liver microsomes and monoclonal antibodies that inhibit specific CYP450 enzymes were used in combination with liquid chromatography-mass spectrometry to identify the enzymes responsible for these transformations. CYP2C19 was found to catalyze the formation of both *cis* and *trans* alcohols of the prenyl side chain of 8-prenylnarigenin with K_m values of 14.8 ± 3.2 µM and 16.6 ± 4.6 µM, respectively. CYP2C8 converted 8-prenylnarigenin regioselectively to the *trans* alcohol of the prenyl group with a K_m of 3.7 ± 0.9 µM. Finally, CYP1A2 was found to catalyze the *O*-demethylation of isoxanthohumol to generate 8-prenlynarigenin, with a K_m value of 17.8 ± 3.7 µM. These results suggest that the estrogenicity of hop constituents in vivo will depend in part upon metabolic conversion that might show individual variation.

Introduction

The female flowers of hops (*Humulus lupulus* L.) are used in the brewing of beer, and extracts of these strobiles are under investigation as possible alternatives to conventional estrogen replacement therapy for menopausal women (Liu et al., 2001). Alternatives to estrogen supplements like Premarin™ and Prempro™ are needed due to safety problems associated with these products such as increased risk of breast cancer, heart diseases and dementia (Chlebowski et al., 2003; Culhane, 2003; Manson et al., 2003; Shumaker et al., 2003; Cushman et al., 2004). The prenylated flavanones in hops include xanthohumol, isoxanthohumol (IX), 8-prenylnaringenin (8PN), and 6-prenylnaringenin (Stevens et al., 1997). Among these, 8PN is the most estrogenic (Milligan et al., 1999; Milligan et al., 2002).

The estrogen receptor binding activity of 8PN has been studied in vitro using the recombinant human estrogen receptors α and β and found to be similar to estradiol (Milligan et al., 2002). A mammalian cell-based transient transactivation assay demonstrated that 8PN is a potent estrogen receptor-α selective agonist (Schaefer et al., 2003). In addition, the estrogenic activities of 8PN have been confirmed using *in vivo* assays of vaginal and uterine mitosis and uterotrophic response (Milligan et al., 2002).

A prenylated flavanone with a methoxy group in the 5 position (see structure in Figure 1), IX is approximately 10-fold more abundant than 8PN in hops and hop extracts (Coldham and Sauer, 2001). In addition, IX can be metabolized by human liver microsomes to form the more potent estrogen 8PN (Nikolic et al., 2005). Although IX has been reported to be cytotoxic and to inhibit the proliferation of the breast cancer cell line, MCF-7 (Miranda et al., 1999), IX was not found to be estrogenic in mice (Milligan et

al., 2000). Overk et al. (2005) have also investigated the relative estrogenicities of IX and 8PN and reported that 8-PN but not IX can activate the estrogen response element in Ishikawa cells, can significantly induce estrogen response element-luciferase expression in MCF-7 cells, and can up-regulate progesterone receptor mRNA in the Ishikawa cell line. However, both 8-PN and IX can up-regulate progesterone receptor mRNA in the MCF-7 cell line. Therefore, 8PN has been shown to be more estrogenic than IX both in vitro and in vivo.

During previous studies of in vitro metabolism of 8PN using human liver microsomes (Nikolic et al., 2004; Nikolic et al., 2005), the most abundant human liver metabolites were identified as oxidation products of the prenyl group. Hydroxylation was found to occur at the terminal methyl groups to produce *cis* and *trans* alcohols (see Figure 1). The *trans* alcohol of 8PN, 8PN-M2, was the most abundant metabolite of 8PN. In addition to 8PN, metabolism of IX by human liver microsomes produced the hydroxylated prenyl side chain metabolites IX-M1 and IX-M2 (see structures in Figure 1).

In this investigation, the cytochrome P450 enzymes which catalyze the oxygenation of the prenyl side of IX and 8PN in vitro were identified using monoclonal antibody inhibitors of specific enzymes as well as chemical inhibitors of CYP450 enzymes. In addition, the kinetics of the formation of 8PN-M1 (the *cis* alcohol of the terminal prenyl methyl group of 8PN) from 8PN and the formation of 8PN from IX were determined using recombinant human cytochrome P450 enzymes. The cytochrome P450 enzymes which contribute to the *O*-demethylation of isoxanthohumol were also identified. The identification of the cytochrome P450 enzymes involved in the formation of significant metabolites of IX and 8PN should be useful in predicting potential hop-

drug interactions and whether significant variation in the metabolism of IX and 8PN might occur in the population due to genetic variation.

Methods

Chemicals, enzymes and antibodies

Racemic 8PN and IX were isolated from hops and purified by semi-preparative HPLC as described previously (see Nikolic et al., 2004 and Chadwick et al., 2004, respectively). 8PN-M1 and 8PN-M2 were synthesized as described by Nikolic et al. (2004), and IX-M2 was isolated as described by Chadwick et al. (2004). Purity was determined to be >98% based on HPLC and LC-MS analysis. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA).

Pooled human liver microsomes were purchased from In Vitro Technologies (Baltimore, MD). The total cytochrome P450 content of the microsomes was 0.17 nmol/mg of protein. Anti-CYP 1A1, 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C9*2, 2C19, 2D6, 2E1 and 3A4, monoclonal antibodies were obtained from Dr. Harry Gelboin of the National Institutes of Health (Belthesda, MD). Protein concentrations of the liver microsomes and the monoclonal antibodies were determined by using the method of Bradford (1976) with bovine serum albumin as a standard. Microsomes from baculovirus-infected insect cells containing human cytochrome P450 reductase and human cytochrome b_5 with cDNA-expressed human CYP2C8 (1.0 nmol cytochrome P450/mL), CYP 2C19 (1.0 nmol cytochrome P450/mL) or CYP1A2 (1.0 nmol cytochrome P450/mL), were purchased from BD Biosciences (San Jose, CA).

Linearity assays

In preparation for inhibition and kinetics assays, the linearity of the formation of each major metabolite including 8PN-M1, 8PN-M2, IX-M1, IX-M2, and 8PN (from IX) was investigated by incubating 8PN or IX (10 μM) with human liver microsomes (0.5 mg/mL). The reactions were stopped at various time points up to 40 min, and the major metabolites were measured using LC-MS as described below. The formation of 8PN-M1, 8PN-M2, IX-M1, and IX-M2 was linear up to 40 min (data not shown). However, the formation of 8PN from IX was linear only during the first 15 min (data not shown).

Inhibition of cytochrome P450 enzymes

To identify specific cytochrome P450 enzymes responsible for the formation of significant metabolites of IX and 8PN, incubations were carried out using either monoclonal antibodies or chemical inhibitors of specific enzymes. All incubations contained 10 μM 8PN or IX, 1 mg/mL of human hepatic microsomal protein, and 1 mM NADPH in 50 mM phosphate buffer at pH 7.4 in a total volume of 0.4 mL. Either monoclonal antibody inhibitors of specific cytochrome P450 enzymes were added at a final concentration of 0.4 mg/mL, or chemical inhibitors were used. The chemical inhibitors consisted of either 10 μM omeprazole, 20 μM quercetin, 10 μM furafyline, or 10 μM sulfaphenazole (Becquemont et al., 1999; Kim et al., 2004), which selectively inhibited CYP2C19, CYP2C8, CYP1A2, or CYP2C9.

Each enzymatic reaction was preceded by a 5 min preincubation and was initiated by the addition of 10 mM NADPH. Control incubations were carried out that contained all components except the inhibitory antibodies or compounds. All of these incubations were carried out at 37 °C for 30 min, except for the IX *O*-demethylation

reaction, which was incubated for 10 min (based on the preliminary linearity studies). Reactions were terminated by the addition of 1.6 mL of ice cold acetonitrile/ethanol (1:1, v/v). After centrifugation to remove the precipitated proteins, the supernatants were removed and evaporated to dryness under vacuum. Each residue was reconstituted in 150 µL of HPLC mobile phase immediately prior to analysis using liquid chromatography-mass spectrometry (LC-MS). All incubations using inhibitory antibodies or chemical inhibitors were carried out at least three times, means were calculated, and the mean values were compared using one-way ANOVA with Tukey-test, $p \leq 0.01$.

It should be note that prior to beginning the IX and 8PN studies, the inhibitory activity of the monoclonal antibodies was verified by incubating the human liver microsomes (protein concentration of 25 mg/mL) with or without 10 µM of each of the following standard chemical substrates: dextromethorphan, chlorzoxazone, amodiaquine, phenacetin, tolbutamide, (S)-mephenytoin, or testosterone. These compounds are substrates for CYP2D6, CYP2E1, CYP2C8, CYP1A2, CYP2C9, CYP2C19, and CYP3A4, respectively. After 30 min of incubation at 37 °C, the formation of the following metabolites was measured using LC-MS: dextrorphan, 6-hydroxychlorzoxazone, desethylamodiaquine, acetaminophen, hydroxytolbutamide, 4'-hydroxy-(S)-mephenytoin, and 6β-hydroxytestosterone, respectively. The monoclonal antibodies inhibited these reactions by 90, 90, 85, 90, 80, 100, and 86%, respectively, which was adequate for the identification of enzymes that inhibited the metabolism of IX and 8PN.

Assays using recombinant cytochrome P450 enzymes

To confirm that the cytochrome P450 enzymes identified using the inhibition screening assays catalyzed the formation of the expected 8PN or IX metabolites and to investigate the relative contribution of each enzyme, additional incubations were carried out using recombinant CYP2C8, CYP2C19 or CYP1A2. Each reaction contained 10 µM 8PN or IX, 1.0 mM NADPH, and 1 nmol/mL of CYP2C8, CYP2C19, or CYP1A2 (containing co-expressed P450 reductase and cytochrome b_5). Incubations were carried out for 10 min each at 37 °C.

The measurement of the kinetics of the formation of 8PN-M1 and 8PN-M2 from 8PN catalyzed by human liver microsomes has been described previously (see Nikolic et al., 2004). Briefly, 37.5 pmol/mL of CYP2C8 or 10 pmol/mL of CYP2C19 was incubated with 8PN at 0.625 to 100 µM. Each incubation was carried out at 37 °C for 10 min. The kinetics of 8PN formation was also studied using 12.5 pmol/mL of CYP1A2 and IX at concentrations from 0.625 to 100 µM. The reactions were terminated by the addition of cold acetonitrile/ethanol and prepared for LC-MS analysis as described above for the inhibition studies. Quantitative analyses of 8PN-M1, 8PN-M2, and 8PN were carried out using LC-MS as described below. Curve fitting of the kinetics data (Michaelis-Menten and Eadie-Hofstee plots) and the K_m and the V_{max} were calculated using SigmaPlot 8.0 software (Richmond, CA).

LC-MS analysis

The formation of specific metabolites of IX and 8PN was determined by comparison with authentic standards using LC-MS with reversed phase HPLC separation and negative ion electrospray mass spectrometric detection. In addition, quantitative analyses of the major metabolites of IX and 8PN were carried out using LC-

MS. Standard curves for these compounds were prepared using either synthetic standards or compounds isolated from hops. The LC-MS system consisted of an Agilent (Palo Alto, CA) 1100 HPLC system interfaced to a G1946A quadrupole mass spectrometer. HPLC separations were carried out using a YMC (Wilmington, NC) ODS-AQ S-3 reversed phase column (2.0 x 150 mm; 5 µm particle size) with a 30 min linear gradient from 35 to 70% methanol in 0.05% aqueous acetic acid followed by a 10 min gradient from 70 to 90% methanol, and then 90 to 95% methanol in 5 min. The column was re-equilibrated for 8 min between injections. The flow rate was 0.2 mL/min, the column temperature was 30 °C, and the autosampler was maintained at 4 °C. During negative ion electrospray, the capillary voltage was 2.5 kV, the flow rate of the drying gas was 5.0 L/min, and the nebulizer gas pressure was 40 psig. Selected ion monitoring with a dwell time of 115 msec/ion was used to monitor the deprotonated molecules of 8PN (*m/z* 339.1), 8PN-M1 and 8PN-M2 (*m/z* 355.1), IX-M1 and IX-M2 (*m/z* 369.1), and the internal standard, naringenin (*m/z* 271.1).

Results

Metabolism of 8PN by human cytochrome P450 enzymes

Incubations of 8PN and human liver microsomes were carried out with each of 11 different monoclonal antibodies that inhibit specific cytochrome P450 enzymes in order to identify enzymes responsible for the formation of the most abundant metabolites 8PN-M1 and 8PN-M2. The cytochrome P450 enzymes inhibited by these monoclonal antibodies represent more than 75% of the cytochrome P450 enzymes in the human

liver. The results of these enzyme inhibition studies for 8PN metabolism are summarized in Figure 2.

As shown in Figure 2, CYP2C19 and CYP2C8 were primarily responsible for the formation of 8PN-M1 and 8PN-M2. In particular, only CYP2C19 catalyzed the formation of the *cis* alcohol, 8PN-M1, while both CYP2C19 and CYP2C8 formed the *trans* alcohol, 8PN-M2. These results were confirmed by the use chemical inhibitors of cytochrome P450 enzymes (see Figure 3). For example, incubation of omeprazole, which inhibits CYP2C19, with human liver microsomes resulted in a significant reduction in formation of both 8PN-M1 and 8PN-M2. Treatment of the human liver microsomes with quercetin, which is an inhibitor of CYP2C8, but not CYP2C19, resulted in no reduction in the formation of 8PN-M1 as expected. However, no significant reduction in the formation of 8PN-M2 was observed either, although there was a downward trend (Figure 3). This marginal inhibitory effect of quercetin on CYP2C8 was also reported by Desta et al. (2000) when studying the metabolism of cisapride.

As independent confirmation of the results from the use of selective enzyme inhibitors for the identification of cytochrome P450, recombinant forms of the enzymes responsible for the formation of 8PN-M1 and 8PN-M2, CYP2C8 or CYP2C19, were incubated with 8PN, and the metabolites were characterized using LC-MS (see Figure 4). These studies showed that CYP2C8 can form trace amounts of 8PN-M1 (note that the signal for 8PN-M1 was significantly more than that of the control without enzyme, $p < 0.05$). Furthermore, the formation of 8PN-M2 by CYP2C8 was more efficient than that of 8PN-M1 (see Figure 4). As indicated by the inhibition experiments, both CYP2C8 and CYP2C19 catalyze the formation of 8PN-M2 with the contribution of CYP2C19

predominating approximately 3.5-fold, based on the comparison of V_{max}/K_m for both enzymes (see Table 1). Overall, CYP2C8 showed greater stereoselectivity and favored the formation of 8PN-M2 over 8PN-M1 (see Figure 4).

Preliminary experiments were carried out to determine the linearity of 8PN-M1 and 8PN-M2 production with respect to the concentration of human CYP2C8 and CYP2C19 protein. The rate of formation of 8PN-M2 was linear for at least 10 min during the incubation of CYP2C8 (at 12.5, 25, and 50 nM) with 10 µM 8PN (data not shown). In addition, the formation of both 8PN-M1 and 8PN-M2 from 10 µM 8PN was linear for at least 10 min during incubations with human CYP2C19 (at 3.12, 6.25, and 12.5 nM) (data not shown). Therefore, 37.5 nM CYP2C8 and 10 nM CYP2C19 were incubated with 10 µM 8PN for 10 min during all subsequent experiments.

The kinetics of the formation of 8PN-M1 and 8PN-M2 catalyzed by human CYP2C8 and CYP2C19 were determined and are shown in Figure 5 and Table 1. The kinetics parameters were calculated using a non-linear regression analysis fit of the data to the Michaelis-Menten equation. The K_m values (the apparent affinity constants) for the metabolism of 8PN to form 8PN-M1 and 8PN-M2 by CYP2C19 were 14.8 µM and 16.6 µM, respectively. Despite the similarity of K_m values, the maximum initial enzyme velocity, V_{max}, for the formation of 8PN-M2 from 8PN was approximately two-fold greater than the V_{max} for the formation of 8PN-M1 by CYP2C19. This resulted in a specificity constant (V_{max}/K_m) for the formation of 8PN-M2 by CYP2C19 that was twice that of 8PN-M1. The kinetics of 8PN-M2 formation from 8PN by CYP2C8 was also determined, and the V_{max} and K_m were both less than the corresponding values for 8PN-

M2 formed by CYP2C19 (see Table 1). However, the specificity constant was approximately 30% that of CYP2C19 formation of 8PN-M2 from 8PN.

The kinetics data for the formation of 8PN-M1 and 8PN-M2 from 8PN by pooled human liver microsomes were redrawn as Eadie-Hofstee plots. As indicated by these plots in Figure 6, the formation of both 8PN-M1 and 8PN-M2 was non-linear. The implications of this non-linearity are discussed below.

Metabolism of IX by human cytochrome P450 enzymes

Monoclonal antibodies that inhibit specific cytochrome P450 enzymes were also used to identify enzymes that catalyze the formation of the *cis* and *trans* alcohols of the terminal prenyl methyl group of IX as well as the *O*-demethylation of IX to form 8PN. The results of these incubations and LC-MS assays are shown in Figure 7 and indicate that CYP2C19 is primarily responsible for the formation of the hydroxylated metabolite IX-M2. After 30 min of incubation of IX with human liver microsomes and an anti-CYP2C19 monoclonal antibody, the formation of IX-M2 and IX-M1 was reduced 94% and 77%, respectively, compared to their formation in a control incubation that was identical except for the omission of the inhibitory antibodies. Therefore, CYP2C19 was the most active human cytochrome P450 enzyme in the generation of both IX-M2 and IX-M1.

Next, the cytochrome P450 enzymes catalyzing the *O*-demethylation of IX to 8PN were identified by using monoclonal antibody inhibitors and then confirmed by using a chemical inhibitor. As shown in Figure 7A, CYP1A2 was found to be the enzyme responsible for the formation of 8PN from IX based on the use of monoclonal antibody inhibitors. Subsequently, treatment of human liver microsomes with 10 μM

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furafyline, which is a selective inhibitor of CYP1A2, reduced the *O*-demethylation of IX by 93%, compared with control which contained all ingredients except the inhibitory compounds (data not shown). Therefore, CYP1A2 was identified as the enzyme primarily responsible for the formation of 8PN from IX.

These results for the formation of IX metabolites were confirmed by incubating IX with recombinant human CYP1A2 and CYP2C19. As shown in Figure 8, CYP1A2 catalyzed the *O*-demethylation of IX to form 8PN, and CYP2C19 catalyzed the hydroxylation of IX to IX-M1 and IX-M2. Furthermore, the formation of IX-M2 from IX by CYP2C19 was favored slightly over formation of IX-M1 (see Figure 8B).

The kinetics of the formation of 8PN catalyzed by human CYP1A2 was investigated as shown in Figure 9 and Table 1. The kinetic data were calculated using a non-linear regression analysis to fit the parameters of the Michaelis-Menten equation. The K_m value for the metabolism of IX to form 8PN was determined to be 17.7 μM.

Discussion

In addition to use during the brewing of beer, hop extracts are under investigation as botanical dietary supplements for the relief of hot flashes in menopausal women due to their estrogenic properties (Liu et al., 2001). Although the most estrogenic constituent of hop preparations is 8PN, it is much less abundant than xanthohumol or isoxanthohumol (Coldham and Sauer, 2001). However, xanthohumol is known to cyclize to form the weak estrogen IX under the acidic conditions of the stomach, and IX can be metabolically *O*-demethylated to form the potent estrogen 8PN (Nikolic et al., 2005). Therefore an understanding of the cytochrome P450 enzymes responsible for

the formation of 8PN from IX would be important for the interpretation of in vivo data from safety and efficacy studies. Also, the enzymes that catalyze the metabolic transformation of 8PN and IX to other specific (perhaps less estrogenic) metabolites should be identified. This information could be used to investigate potential drug-drug interactions or effects of cytochrome P450 polymorphisms that might affect the safety and efficacy of dietary supplements containing hops or hop extracts.

As alternatives to estradiol or equine estrogen preparations such as Premarin™ for possible use by women in the management of menopausal symptoms, the activities of hop preparations containing 8PN, IX, and xanthohumol should be evaluated in vivo as well as in vitro. The in vitro studies reported here concern the identification of enzymes responsible for significant phase I metabolism pathways of 8PN and IX. Although these data will be useful during the design and interpretation of future in vivo studies, additional in vitro studies are required and are in progress in our laboratory to address other factors affecting the bioavailability and efficacy of hop components such as intestinal absorption, enzyme inhibition, and phase II conjugation including glucuronidation and sulfation.

Additional issues that should be investigated concerning the in vivo activities of hop preparations include the evaluation of their selectivity towards estrogen receptor-α as compared to estrogen receptor-β, and their potential antiestrogenic effects. For example, selective estrogen receptor modulators such as tamoxifen (Fisher et al. 1998) are antiestrogens that are useful for the chemoprevention of breast cancer. Therefore, complex preparations such as hop extracts have the potential to contain both estrogenic

and antiestrogenic constituents that might help relieve menopausal symptoms such as hot flashes in women while not contributing to increased risks of breast cancer.

The most abundant oxygenated metabolites of IX and 8PN are the hydroxylated products IX-M1, IX-M2, 8PN-M1, and 8PN-M2. Among these *cis* and *trans* hydroxylated prenyl groups, the *trans* isomers predominate, which is probably due to their lower internal energy and therefore greater stability compared to the *cis* isomers. In this investigation, CYP2C19 was determined to be the cytochrome P450 enzyme that was primarily responsible for the formation of the *trans*-hydroxylated prenyl metabolites of 8PN and IX, 8PN-M2 and IX-M2, respectively. Although CYP2C19 also catalyzed the hydroxylation of 8PN to form the *cis* isomer, 8PN-M1, the specificity constant for the formation of 8PN-M2 by CYP2C19 was 2-fold greater than for 8PN-M1 (Table 1). The formation of 8PN-M2 was also stereoselectively catalyzed by CYP2C8 (Table 1), which was probably the result of the unique spatial arrangement of CYP2C8 active site amino acid residues, resulting in substrate selectivity and regioselectivity (Kerdpin et al., 2004).

The contributions of the enzymes CYP2C19 and CYP2C8 to the formation of 8PN-M2 is consistent with the Eadie-Hofstee plot shown in Figure 6 which was obtained by incubation of 8PN with pooled human liver microsomes. Although the plot for 8PN-M2 formation in Figure 6 is consistent with a model describing the action of more than one enzyme, an alternative explanation that is consistent with these data would be a single enzyme containing more than one binding site. The Eadie-Hofstee plot for the formation of 8PN-M1 in Figure 6 also indicates that either more than one enzyme or more than one binding site might be involved, even though the enzyme inhibition studies showed that only CYP2C19 catalyzed the formation of 8PN-M1 and IX-M1.

Our studies indicate that CYP2C19 and CYP2C8 are responsible, at least in part, for the hydroxylation of the prenyl side chain of 8PN and IX, and CYP1A2 catalyzes the *O*-demethylation of IX to form 8PN. The *cis* and *trans* alcohols of the prenyl side chains of 8PN and IX are the most abundant phase I metabolites of these compounds, and the formation of 8PN from IX has the potential to enhance the estrogenicity of IX. The enzymes CYP1A2 (Saito et al., 2005), CYP2C19 and CYP2C8 (Smith et al., 1998) exhibit polymorphisms in humans. Since these variants sometimes show much lower activities than the wild-type enzymes (e.g., variants of human CYP1A2 have been reported with less than 1% of the wild-type activity), such polymorphisms can be significant sources of inter-individual differences in drug metabolism (Ingelman-Sundberg, 2002). Furthermore, the activities of these enzymes can be regulated by both environmental and genetic factors. Examples of environmental factors that can induce or inhibit the activities of cytochrome P450 enzymes include many prescription drugs, diet, alcohol consumption, and smoking (Crankshaw and Hines, 1992; Zhang et al., 2002; Czekaj et al., 2005). In conclusion, these results suggest that the estrogenicity of hop constituents will depend in part upon metabolic conversion that might show individual variation.

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Unnumbered Footnote

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Figure Legends

Figure 1. *O*-Demethylation of isoxanthohumol (IX) by human cytochrome P450 enzymes leads to the formation of 8-prenylnaringenin (8PN), and oxidation of the terminal methyl group of the prenyl side chain of IX or 8PN results in the formation of *cis* (IX-M1, 8PN-M1) or *trans* (IX-M2, 8PN-M2) alcohols.

Figure 2. Inhibition of 8PN metabolism by monoclonal antibodies (mAbs) that inhibit the activity of specific isozymes of cytochrome P450s. Monoclonal antibodies were incubated with 8PN (10 µM) and human liver microsomes (0.5 mg/mL) at 37 ^{o}C for 10 min. The metabolites A) 8PN-M1 and B) 8PN-M2 are expressed relative to a control incubation containing no antibodies. **Significant inhibition compared to the control (HLM) and determined using one-way ANOVA with the Tukey test, $p \leq 0.01$ (Mean ± Std Error; $N = 3$).

Figure 3. Effects of quercetin (inhibitor of CYP2C8, 20 µM) and omeprazole (inhibitor of CYP2C19, 10 µM) on the formation of the hydroxylated 8-PN metabolites, A) 8PN-M1; and B) 8PN-M2, by human liver microsomes. Control incubations contained either no chemical inhibitor (HLM), no human liver microsomes (no HLM), or no NADPH. **Indicates significant inhibition compared to the HLM control containing no chemical inhibitor as determined using one-way ANOVA with Tukey-test, $p \leq 0.01$ (Mean ± Std Error; $N = 3$).

Figure 4. Relative formation of 8PN metabolites by recombinant CYP2C8 and CYP2C19 (at 1 nmol/mL). Recombinant human P450 enzymes were incubated with 8PN (10 μM) at 37 °C for 10 min in the presence of the NADPH (1 mM). A) 8PN-M1 and 8PN-M2 formation catalyzed by CYP2C8; B) 8PN-M1 and 8PN-M2 formation catalyzed by CYP2C19 (Mean ± Std Error; *N* = 3).

Figure 5. Michaelis-Menten plot of 8PN-M1 and 8PN-M2 formation from 8PN. A) 8PN-M1 and 8PN-M2 formation rate by recombinant human CYP2C19 (the corresponding r^2 values for the curves are 0.97 and 0.98, respectively); and B) 8PN-M2 formation rate by recombinant human CYP2C8 (r^2 = 0.97). Error bars represent the Mean ± Std Dev; *N* = 3.

Figure 6. Eadie-Hofstee plots for the formation of A) 8PN-M1 and B) 8PN-M2 from 8PN by pooled human liver microsomes. Human liver microsomes (0.25 mg/mL of protein) were incubated with 8PN (concentrations ranging from 0.5 to 200 μM) at 37 °C for 10 min.

Figure 7. Inhibition of IX metabolism by monoclonal antibodies (mAb) that inhibit the activity of specific isozymes of cytochrome P450s. A) 8PN formation was measured after incubation of IX with human liver microsomes (0.5 mg/mL) and mAb at 37 °C for 10 min; B) IX-M2 was measured after incubation of IX with human liver microsomes (0.5 mg/mL) and mAb at 37 °C for 30 min; and C) IX-M1 was measured after incubation of IX with human liver microsomes (0.5 mg/mL) and mAb at 37 °C for 30 min. Levels of the

metabolites are expressed relative to a control incubation containing no antibodies. *Significant inhibition compared to the control (HLM) as determined using one-way ANOVA with the Tukey-test, $p \leq 0.05$; ** $p \leq 0.01$ (Mean ± Std Error ; $N = 3$).

Figure 8. Relative formation of IX metabolites by recombinant CYP1A2 or CYP2C19 (at 1 nmol/mL). Recombinant human P450s were incubated with IX (10 µM) at 37 °C for 10 min in the presence of the NADPH (1 mM). A) 8PN formation catalyzed by CYP1A2; B) IX-M1 and IX-M2 formation catalyzed by CYP2C19. **Significant difference between IX-M1 and IX-M2 formation as determined using one-way ANOVA with the Tukey-test, $p \leq 0.01$ (Mean ± Std Error; $N = 3$).

Figure 9. Michaelis-Menten plot of 8PN formation from IX in the presence of recombinant human CYP1A2 (r^2 value for the curve fitting is 0.97). Error bars represent the Mean ± Std Dev; $N = 3$.

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Table 1. Enzymatic constants of 8PN and IX metabolism

	CYP2C8	CYP2C19	CYP1A2
8PN-M1			
V_{max} (pmol/min/pmol)		32.7 ± 2.9 *	
K_m (μM)		14.8 ± 3.2	
Intrinsic clearance (μl/min/pmol)		2.21	
8PN-M2			
V_{max} (pmol/min/pmol)	4.64 ± 0.49*	72.1 ± 8.4	
K_m (μM)	3.72 ± 0.92	16.6 ± 4.6	
Intrinsic clearance (μl/min/pmol)	1.24	4.35	
8PN, (IX *O*-demethylation)			
V_{max} (pmol/min/pmol)			5.47 ± 0.39*
K_m (μM)			17.77 ± 3.74
Intrinsic clearance (μl/min/pmol)			0.31

*Mean ± Std Dev (*N* = 3)

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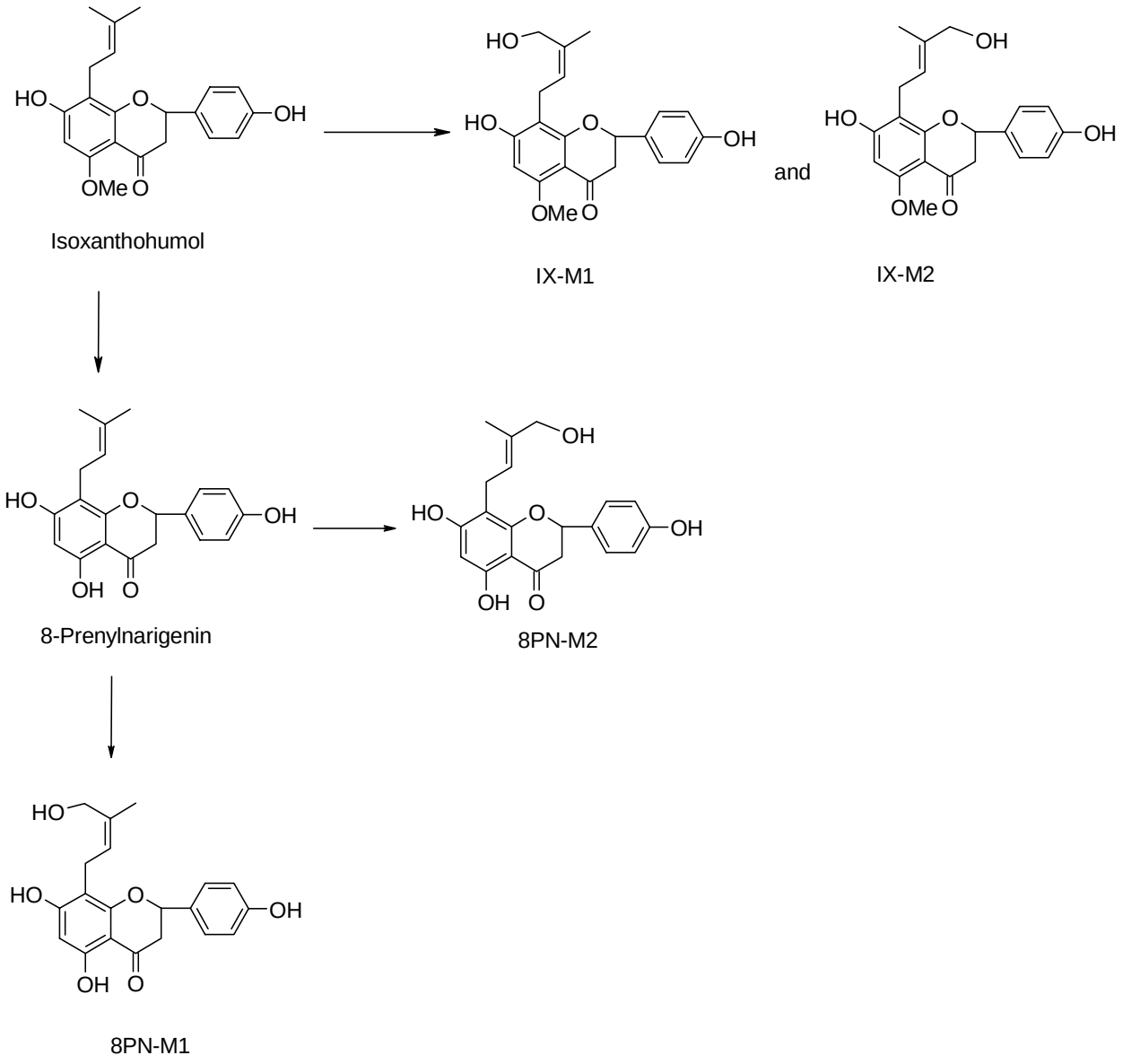

Figure 1

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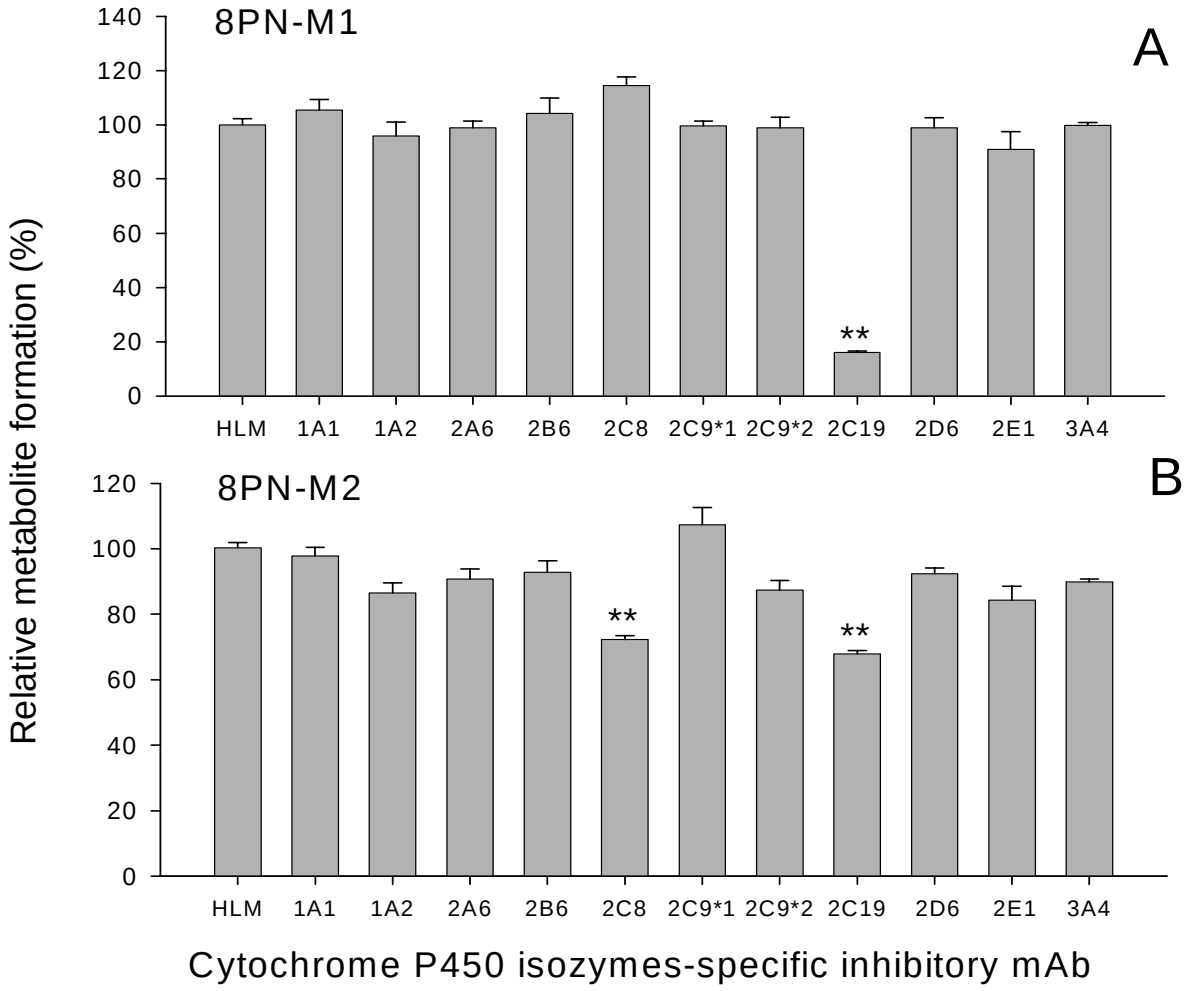

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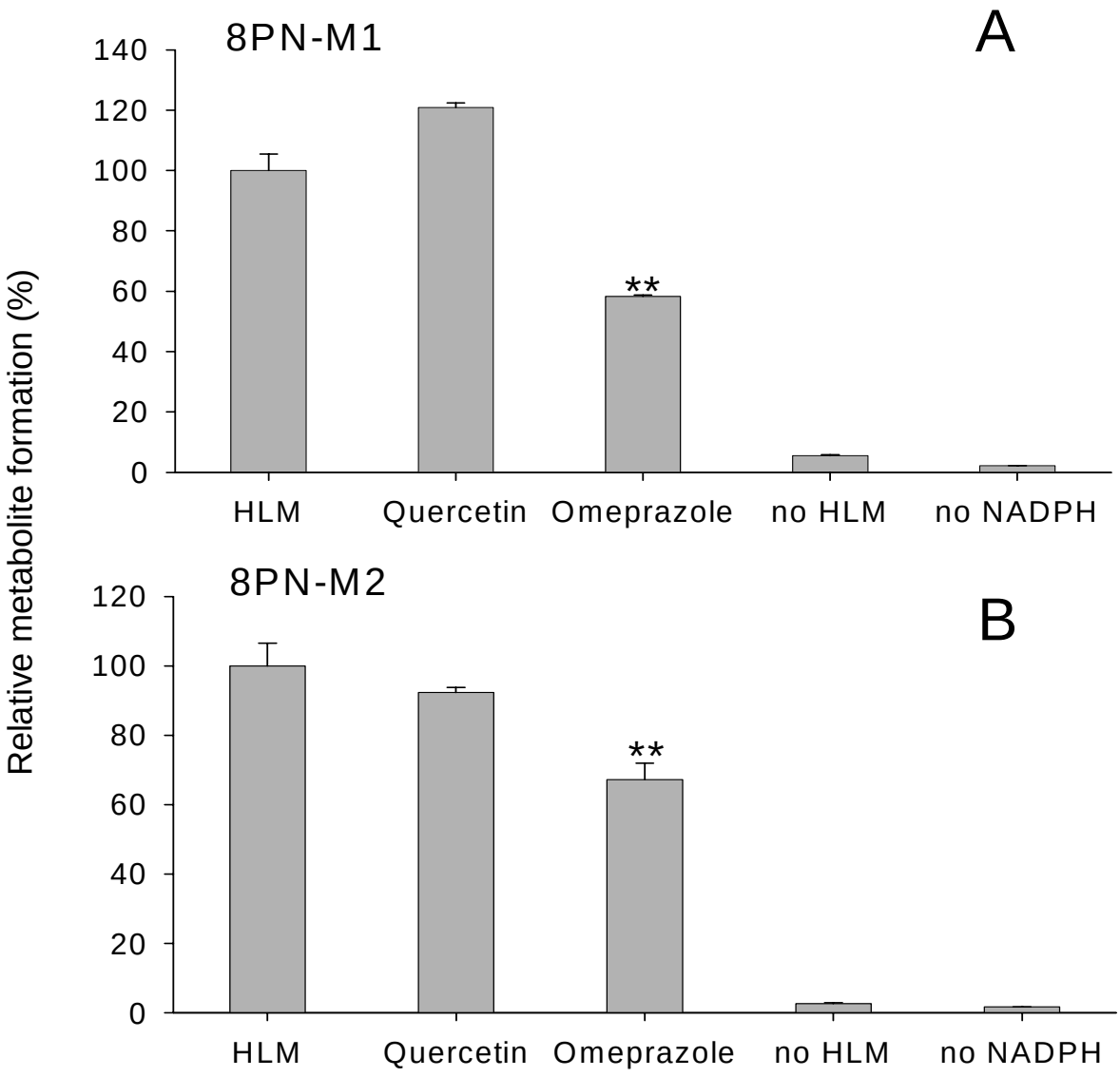

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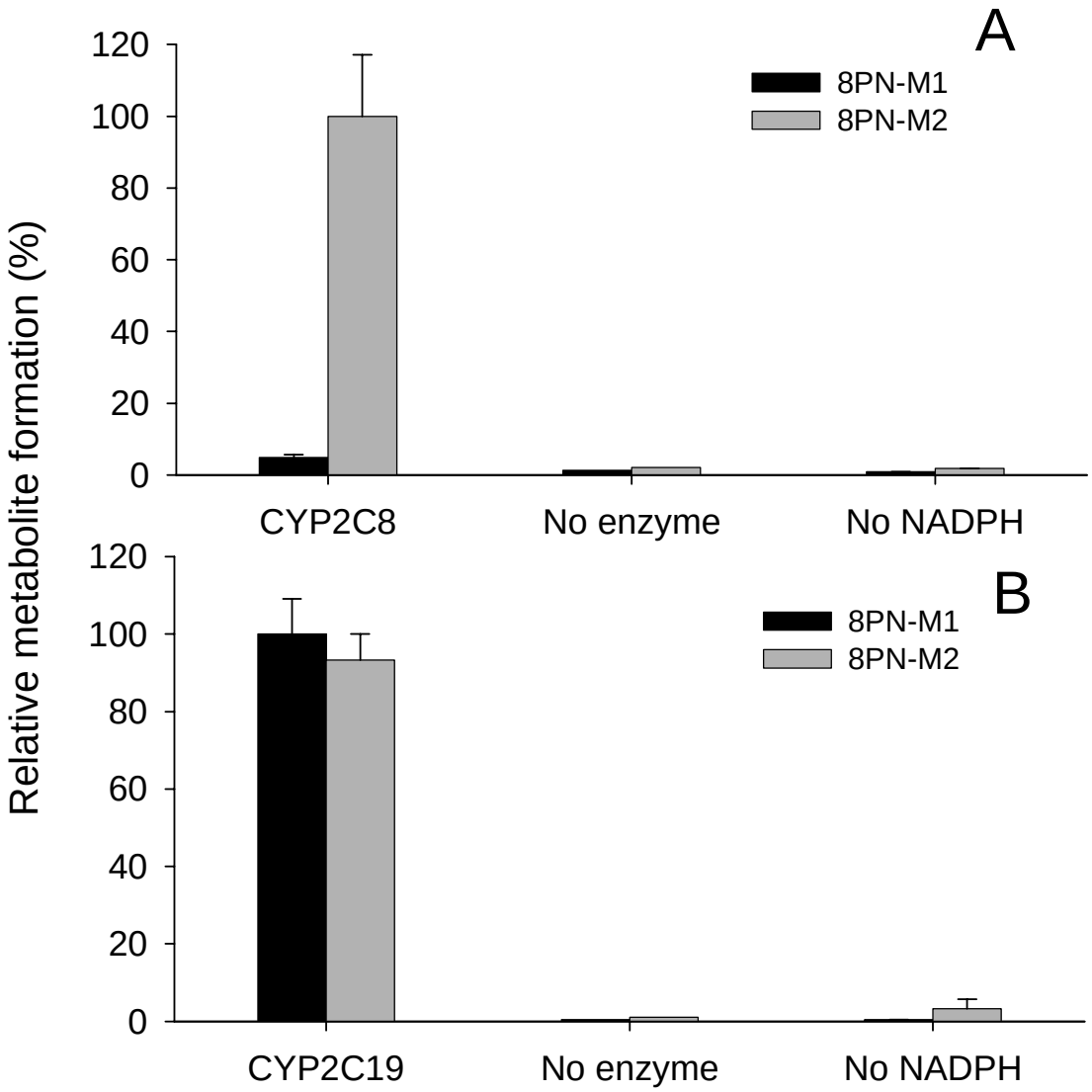

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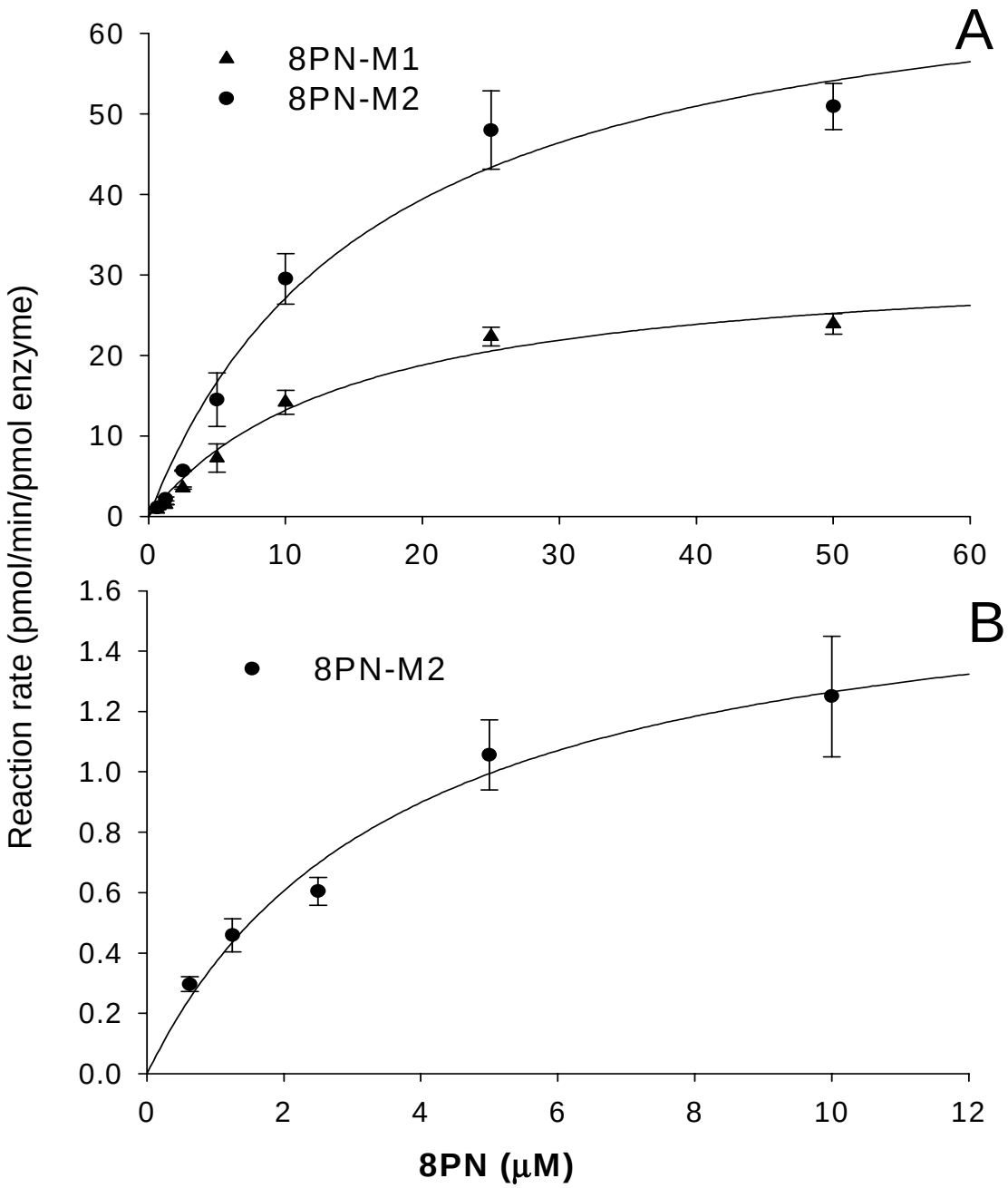

Figure 5

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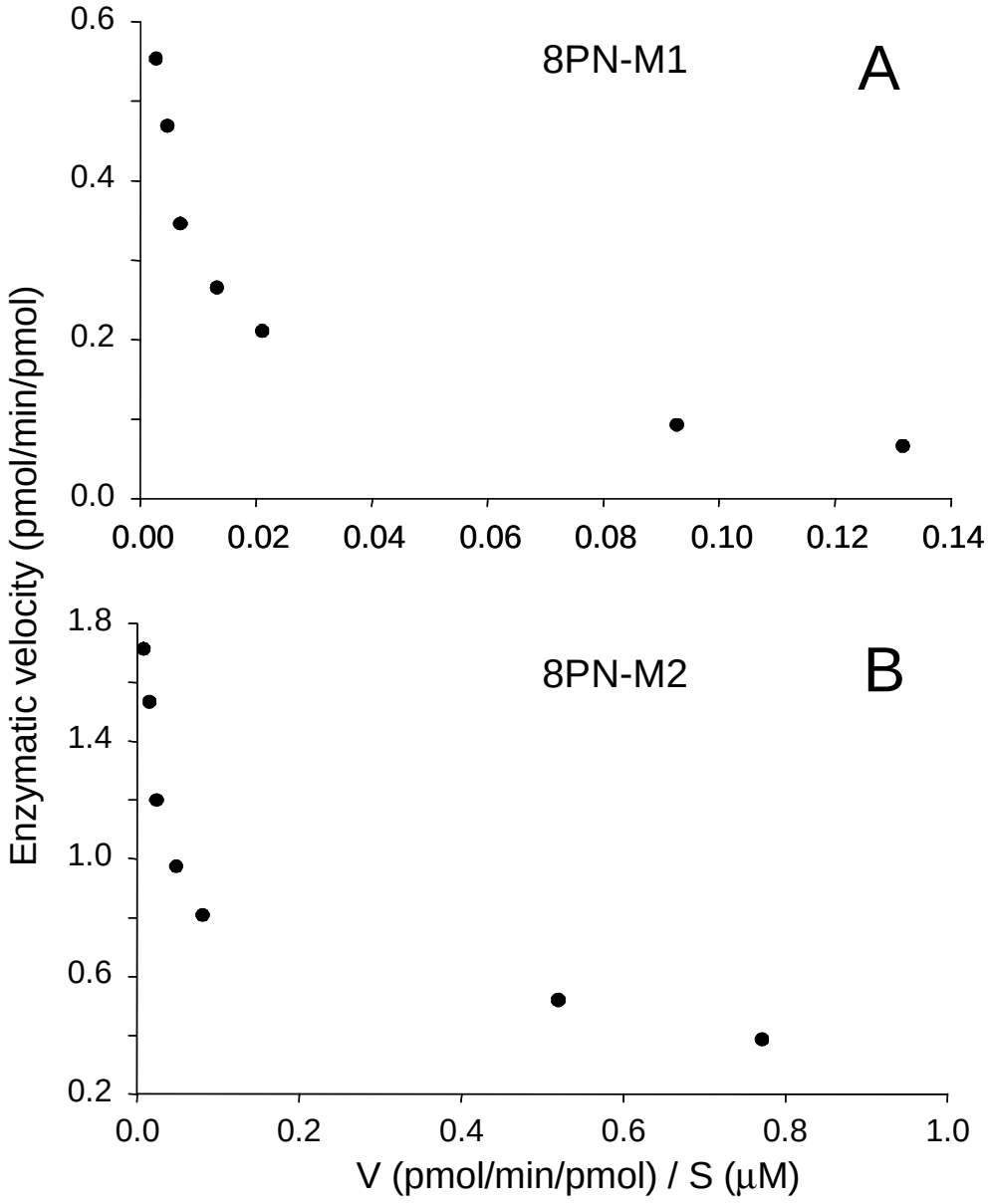

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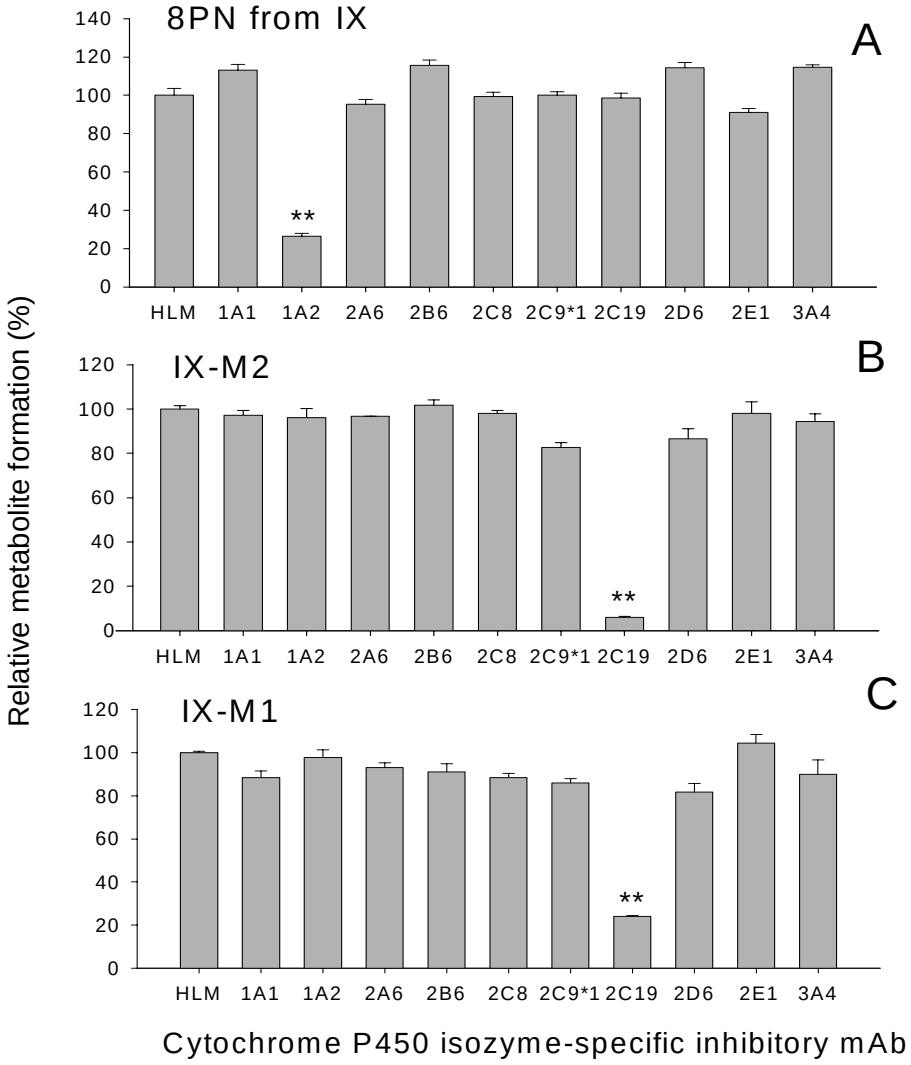

Figure 7

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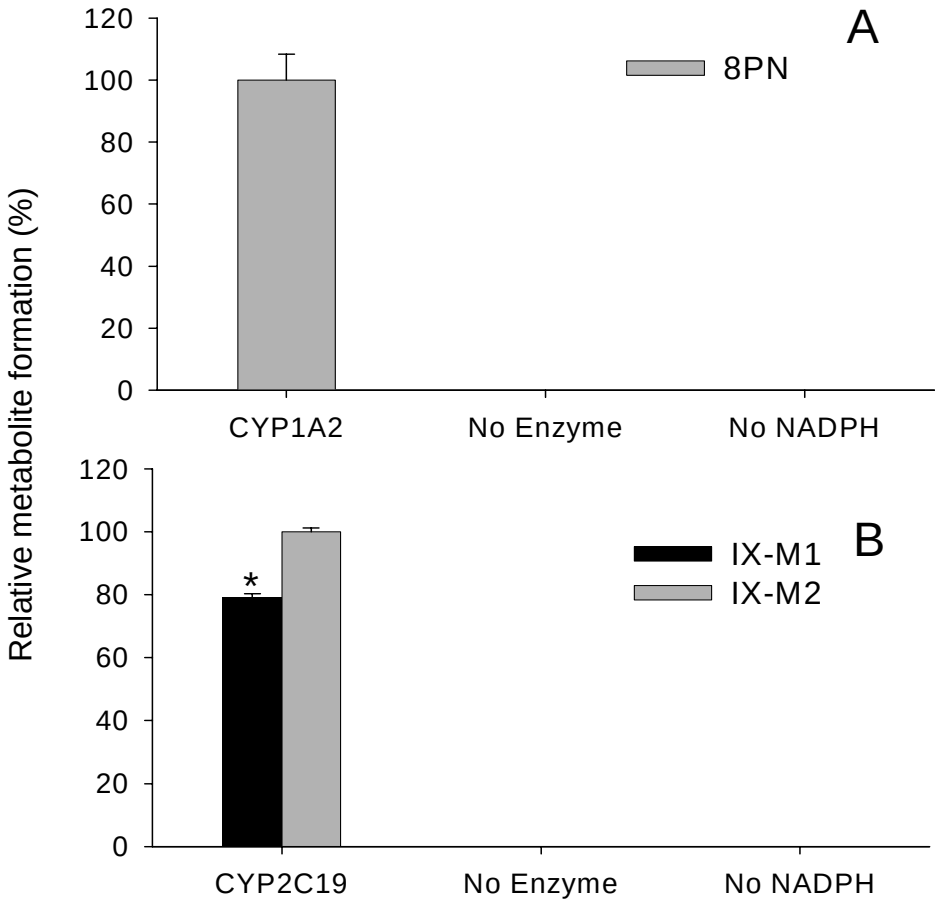

Figure 8

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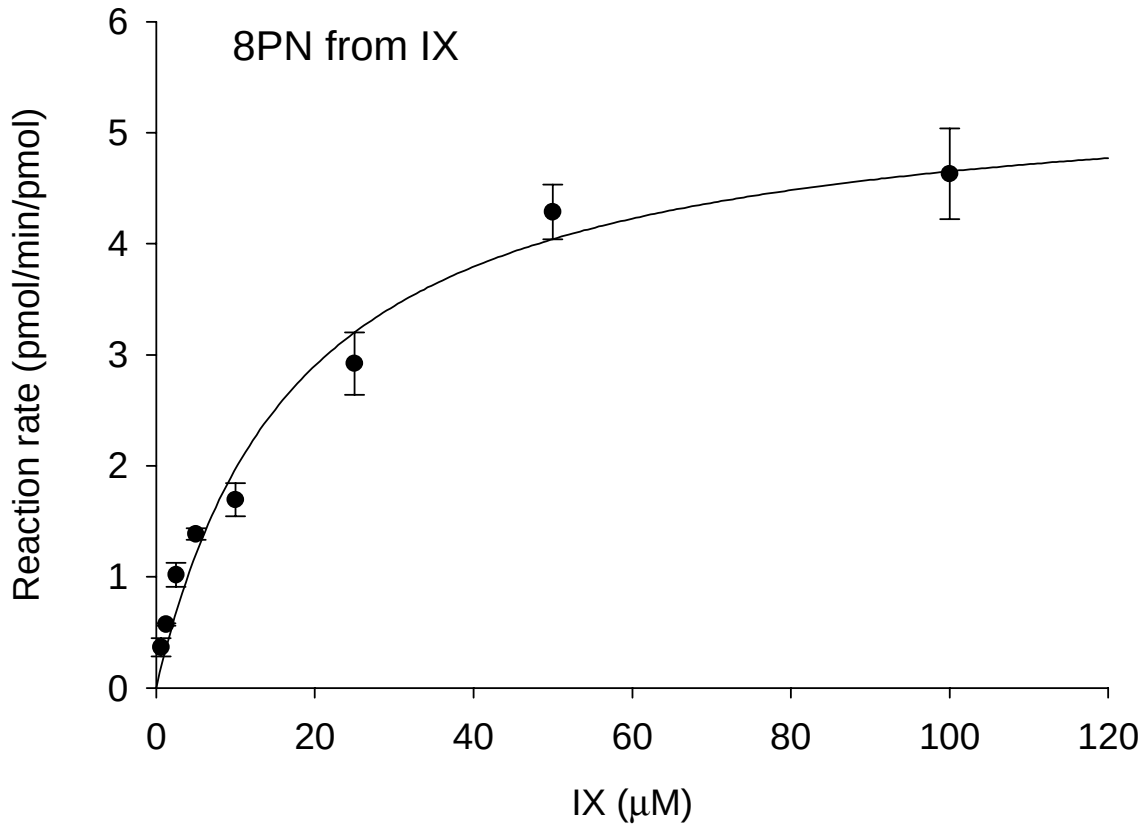

Figure 9