LACK OF EVIDENCE FOR INDUCTION OF CYP2B1, CYP3A23, AND CYP1A2 GENE EXPRESSION BY PANAX GINSENG AND PANAX QUINQUEFOLIUS EXTRACTS IN ADULT RATS AND PRIMARY CULTURES OF RAT HEPATOCYTES

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Running title: Ginseng Extract and Rat P450 Gene Expression

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ABBREVIATIONS: BNF, β-naphthoflavone; BROD, 7-benzyloxyresorufin O-dealkylation; DEX, dexamethasone; DMSO, dimethylsulfoxide; EROD, 7-ethoxyresorufin O-dealkylation; P450, cytochrome P450; PB, phenobarbital; PCR; polymerase chain reaction.
ABSTRACT:

Treatment of rats with a single oral dose (10-30 mg/kg) of a crude *Panax ginseng* extract of unknown ginsenoside content has been reported to modestly increase hepatic microsomal cytochrome P450-mediated aminopyrine *N*-demethylation activity. In the present study, we compared the effect of *Panax ginseng* and *Panax quinquefolius* extracts on rat hepatic CYP2B1, CYP3A23, and CYP1A2 gene expression. Adult male Sprague-Dawley rats (250-275 g) were administered by oral gavage or i.p. with *Panax ginseng* extract (4% w/w total ginsenosides; 30 or 100 mg/kg/day for 1 or 4 days), *Panax quinquefolius* extract (10% w/w total ginsenosides; 100 or 400 mg/kg/day for 21 consecutive days), or an equivalent volume (2 ml/kg) of the vehicle (0.9% NaCl or 0.3% carboxymethylcellulose) and terminated one day after the last dose. *Panax ginseng* and *Panax quinquefolius* extracts did not affect body weight gain, absolute or relative liver weight, hepatic CYP2B1, CYP3A23, or CYP1A2 mRNA expression, or microsomal CYP2B-mediated 7-benzyloxyresorufin *O*-dealkylation (BROD) or CYP1A-mediated 7-ethoxyresorufin *O*-dealkylation (EROD) activity. In contrast, results from positive control experiments indicated that phenobarbital increased CYP2B1 mRNA and BROD activity, dexamethasone increased CYP3A23 mRNA, and β-naphthoflavone increased CYP1A2 mRNA and EROD activity levels. Treatment of primary cultures of rat hepatocytes with either of the ginseng extracts (0.1-1000 µg/ml for 2 days) also did not affect CYP2B1 or CYP3A23 mRNA expression. Overall, our data indicate that *Panax ginseng* and *Panax quinquefolius* extracts do not increase rat hepatic CYP2B1, CYP3A23, or CYP1A2 gene expression.
For more than 3000 years, ginseng has been used in Asia for the treatment of various medical disorders. Since the 18th century, it has been one of the most commonly used herbal medicines in Europe and North America (Soldati, 2000). According to a recent survey, ginseng is the second most popular herbal medicine in the U.S.A. (Barnes et al., 2004). Several types of ginseng have been identified to date, including those in the Panax genus. The best characterized species in this genus are Panax ginseng C. A. Meyer (also known as Asian ginseng) and Panax quinquefolius L. (also known as North American ginseng) (Soldati, 2000).

The impetus for the present investigation is the paucity of information in the scientific literature on the effect of ginseng on hepatic expression of drug-metabolizing enzymes. In a previous study (Lee et al., 1987), the administration of a single oral dose (10, 20, or 30 mg/kg) of a crude Panax ginseng extract to adult male rats resulted in a small but statistically significant increase (18-24%) in hepatic microsomal cytochrome P450 (P450)-mediated aminopyrine N-demethylation activity. In contrast, the same treatment did not increase S-9 aryl hydrocarbon hydroxylation activity in an assay that used benzo[a]pyrene as the substrate. The investigators used a crude extract of unknown ginsenoside content and the effects on individual P450 enzymes were not studied. It is also not known whether another type of ginseng, such as Panax quinquefolius, influences P450 expression.

CYP2B1 and CYP3A23 catalyze aminopyrine N-demethylation (Imaoka et al., 1988), whereas CYP1A2 is a catalyst of aryl hydrocarbon hydroxylation (Ryan and Levin, 1990). Therefore, in the present study, we quantified hepatic CYP2B1, CYP3A23, and CYP1A2 expression in adult male rats administered Panax ginseng or Panax quinquefolius extract of known ginsenoside content. We also determined whether these extracts modulate P450 gene expression in primary cultures of rat hepatocytes.
Materials and Methods

**Chemicals and Reagents.** *Panax ginseng* (G115™, lot no. 1254/485; 4% w/w total ginsenosides) and *Panax quinquefolius* extracts (lot no. AGSP110200; 10% w/w total ginsenosides) were provided by Pharmaton S.A. (Bioggio, Switzerland) and the Canadian Phytopharmaceuticals Corp. (Richmond, British Columbia, Canada), respectively. The amount of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2 present in the *Panax ginseng* and *Panax quinquefolius* extracts have been detailed elsewhere (Chang et al., 2002). 7-Ethoxyresorufin, 7-benzyloxyresorufin, β-naphthoflavone (BNF), dexamethasone (DEX), NADPH, bovine serum albumin, SYBR™ Green I, Williams’ medium E, fetal bovine serum, collagenase (Type IV), trypsin inhibitor (Type II-S), trypan blue, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Phenobarbital (PB) was obtained from Wiler PCCA (London, Ont., Canada). Human recombinant insulin, 10× Dulbecco’s phosphate-buffered saline, 10× Hanks’ balanced salt solution (without sodium bicarbonate and without phenol red), Liver Perfusion Media®, Hepatocyte Wash Media®, penicillin-streptomycin, and L-glutamine were obtained from Invitrogen, Inc. (Burlington, Ont., Canada). Matrigel™ basement membrane matrix was purchased from BD Biosciences Canada (Mississauga, Ont., Canada). Percoll™ was bought from Amersham Biosciences, Inc. (Baie d’Urfe, Quebec, Canada). Bio-Rad Protein Assay Kit was bought from Bio-Rad Laboratories (Hercules, CA). The sources of the PCR primers and the reagents for isolation of total RNA, reverse transcription, quantification of total RNA and total cDNA concentrations, and real-time polymerase chain reaction (PCR) have been detailed elsewhere (Chang et al., 2003).
Treatment of Animals. Adult male Sprague-Dawley rats (250-275 g) were purchased from Charles River Co. (Montreal, Quebec, Canada) and housed in our animal care facility, as described previously (Kuo et al., 2004). Rats were administered by oral gavage with *Panax ginseng* extract (30 or 100 mg/kg) or the vehicle (0.9% NaCl) as a single dose or multiple doses (once daily for 4 consecutive days). In another experiment, rats were administered by oral gavage with *Panax quinquefolius* extract (100 or 400 mg/kg) or the vehicle (0.3% carboxymethylcellulose) once daily for 21 consecutive days. In positive control experiments, rats were treated i.p. with BNF (40 mg/kg once daily for 3 consecutive days), PB (80 mg/kg once daily for 4 consecutive days), or DEX (100 mg/kg once daily for 3 consecutive days). All rats were terminated one day after the last dose and livers were excised.

Isolation, Culture, and Treatment of Rat Hepatocytes. Adult male rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.). Hepatocytes were isolated by a two-step collagenase perfusion method (Seglen, 1993) and cell viability was determined by trypan blue exclusion. Hepatocyte suspension was diluted to $10^6$ cells/ml in serum-free, supplemented Williams’ medium E (which contained 1 nM insulin, 100 nM DEX, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin) and a 3 ml volume was plated onto Matrigel™-coated (Schuetz et al., 1988) culture dishes (60 x 15 mm; Permanox). Cells were then incubated for 4 h in a humidified 37°C incubator in the presence of 5% CO$_2$ and 95% air. Subsequently, the medium was decanted to remove unattached cells and hepatocytes were cultured in serum-free, supplemented Williams’ medium E. At 48 h after plating, cultured hepatocytes were treated for 48 h with *Panax ginseng* extract (0.1-1000 µg/ml), *Panax quinquefolius* extract (0.1-1000 µg/ml), or culture medium (vehicle control). In control experiments, cultured hepatocytes were
treated with PB (100 µM), DEX (10 µM) or 0.1% DMSO (vehicle control). Culture medium was changed daily.

**Isolation of Total RNA and Reverse Transcription.** TriZol was used to isolate total RNA from rat liver or cultured rat hepatocytes. The purity and integrity of each isolated RNA preparation was assessed as described previously (Cheung et al., 2004). Total cellular concentrations were determined using the RiboGreen® RNA Quantitation Kit (Jones et al., 1998). Reverse transcription was performed with SuperScript II reverse transcriptase (Cheung et al., 2004) and total cDNA concentrations were quantified using the PicoGreen® dsDNA Quantitation Kit (Singer et al., 1997).

**PCR Primers.** The specificity of the rat CYP1A2 (Borlak and Thum, 2001), CYP2B1 (Li and Kupfer, 1998), CYP3A23 (Mahnke et al., 1997), and cyclophilin (Morris and Davila, 1996) primers was confirmed by sequencing analysis of the purified amplicons (Cheung et al., 2004). The primers used to amplify CYP2B1 do not amplify CYP2B2, CYP2B3, or the CYP2B pseudogenes (Trottier et al., 1996), CYP2B14P and CYP2B16P. Similarly, the primers used to amplify CYP3A23 do not amplify CYP3A2, CYP3A9, or CYP3A18, and the primers used to amplify CYP1A2 do not amplify CYP1A1.

**Real-time PCR Analysis.** Each 20 µl PCR reaction volume contained 1 unit Platinum® Taq DNA polymerase in 1× PCR reaction buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], 4 mM magnesium chloride, 1 ng cDNA, 200 µM dNTP mix, 0.2 µM each of the forward and reverse primers, 0.25 mg/ml bovine serum albumin, and 2 µl of a 3.3× SYBR™ Green I solution. The cycling conditions for real-time cDNA amplification (LightCycler™, Roche Diagnostics, Mannheim, Germany) were 95°C for 1 s (denaturation), 60°C for 6 s (annealing), and 72°C for 10 s (extension) for CYP1A2, 95°C for 1 s, 56°C for 6 s, and 72°C for 23 s for CYP2B1, 95°C
for 1 s, 60°C for 6 s, and 72°C for 14 s for CYP3A23, and 95°C for 1 s, 56°C for 6 s, and 72°C for 12 s for cyclophilin. In all cases, the initial denaturation was performed at 95°C for 5 min. The real-time PCR assays had a bias of less than 5% and a precision (% CV) was less than 19%. The intra-day variability ranged from 3-8%, and the inter-day variability was from 8-30%.

Preparation of Microsomes, Protein Assay, and 7-Alkoxyresorufin O-Dealkylation Assays. Microsomes were isolated by differential ultracentrifugation (Lu and Levin, 1972). Protein concentrations were determined using the Bio-Rad Protein Assay Kit. EROD and BROD activities were determined as described previously (Kuo et al., 2004).

Statistics. The significance of the difference between the means was assessed by one-way analysis of variance, and where appropriate, was followed by the Student Newman-Keuls test (SigmaStat™ software program, Jandel Scientific Co., San Rafael, CA). The level of significance was set a priori at p < 0.05.

Results and Discussion

In the present study, the oral administration of a single dose of *Panax ginseng* extract of known ginsenoside content (Chang et al., 2002) at a dosage of 30 mg/kg or 100 mg/kg to adult male rats did not affect body weight gain or liver weight (data not shown). The 30 mg/kg dosage was chosen because a previous study indicated that treatment of adult male rats with a single dose at 10-30 mg/kg resulted in a modest increase in a P450-mediated enzyme activity (Lee et al., 1987). However, our real-time PCR analysis showed that *Panax ginseng* extract did not modulate CYP2B1 (Fig. 1A), CYP3A23 (Fig. 1B), or CYP1A2 (Fig. 1C) mRNA expression. The same conclusions were drawn regardless of whether the gene expression data were normalized to the amount of total double-stranded DNA (e.g. Fig. 1A-1C) or to cyclophilin.
mRNA levels (data not shown). There was also a lack of an effect on hepatic microsomal CYP1A-mediated EROD and CYP2B-mediated BROD activities (data not shown). Similarly, oral or i.p. administration of *Panax ginseng* extract at a dosage of 100 mg/kg once daily for 4 consecutive days did not influence hepatic CYP2B1, CYP3A23, or CYP1A2 gene expression (data not shown). In contrast, positive control experiments indicated that PB increased hepatic CYP2B1 mRNA expression (47-fold; Fig. 1A) and microsomal BROD activity (37-fold), DEX increased hepatic CYP3A23 mRNA expression (75-fold; Fig. 1B), and BNF increased hepatic CYP1A2 mRNA expression (20-fold; Fig. 1C) and microsomal EROD activity (19-fold).

Our data obtained with the *in vivo* administration of *Panax ginseng* are consistent with those reported in recent clinical studies. In an open-label, randomized study (Gurley et al., 2002) performed in healthy volunteers (6 males and 6 females), the oral ingestion of *Panax ginseng* (500 mg three times daily, and formulated to contain 5% total ginsenosides) did not modify the group mean 1-h serum ratio of 1-hydroxymidazolam/midazolam, which is used as an *in vivo* index of CYP3A4 activity (Thummel et al., 1994), or the 6-h serum ratio of paraxanthine/caffeine, which is used as an *in vivo* index of CYP1A2 activity (Fuhr and Rost, 1994). In another clinical study (Anderson et al., 2003), the oral ingestion of *Panax ginseng* (Ginsana™, 100 mg twice daily for 14 days, and formulated to contain 4% total ginsenosides) by 12 adult female volunteers did not affect the urinary 6β-hydroxycortisol/cortisol ratio, which is another *in vivo* index of CYP3A4 expression (Galteau and Shamsa, 2003).

*Panax ginseng* and *Panax quinquefolius* differ chemically from each other in several ways: 1) ginsenoside Rf is present in *Panax ginseng* but absent in *Panax quinquefolius* (Li et al., 2000); 2) 24-(R)-pseudoginsenoside F11 is absent in *Panax ginseng* but present in *Panax quinquefolius* (Li et al., 2000); 3) the ratio of ginsenoside Rb1 to ginsenoside Rb2 is greater in
Panax quinquefolius than in Panax ginseng (Ji et al., 2001); and 4) the ratio of ginsenoside Rg1 to ginsenoside Rb1 is less in Panax quinquefolius than in Panax ginseng (Ji et al., 2001). Therefore, not surprisingly, differences have been reported in the magnitude of the effects produced by these two types of ginseng. For example, in our recent study (Chang et al., 2002), Panax quinquefolius extract was 45-fold more potent than Panax ginseng extract in the in vitro inhibition of human CYP1A2 catalytic activity, as determined by a comparison of the apparent \( K_i \) values. In the present investigation, rats were treated orally with Panax quinquefolius extract at a dosage of 100 or 400 mg/kg once daily for 21 consecutive days. This dosage regimen was chosen because a previous study showed that Panax quinquefolius extract administered at a dosage of 100 mg/kg once daily for 14 or 28 days was effective in modulating specific biological responses in rats (Murphy et al., 1998). Our results indicated that Panax quinquefolius extract did not affect body weight gain or liver weight (data not shown). It also did not increase hepatic CYP2B1, CYP3A23, or CYP1A2 gene expression, or hepatic microsomal CYP2B-catalyzed BROD activity or CYP1A-catalyzed EROD activity (data not shown).

To determine directly whether ginseng extract is capable of increasing CYP2B1 and CYP3A23 gene expression, primary cultures of rat hepatocytes were treated for 48 h with Panax ginseng or Panax quinquefolius extract at a concentration of 0.1, 1, 10, 100, or 1000 µg/ml. As indicated in Table 1, these extracts did not increase CYP2B1 or CYP3A23 mRNA levels, whereas control experiments showed PB induction of CYP2B1 mRNA (49-fold) and DEX induction of CYP3A23 mRNA (41-fold) in cultured rat hepatocytes. Overall, the in vitro data indicate that the lack of an effect by the in vivo administration of the ginseng extracts was likely not due to inadequate bioavailability.
In summary, the present study provides the first demonstration in rats and primary cultures of rat hepatocytes that \textit{Panax ginseng} and \textit{Panax quinquefolius} extracts of known ginsenoside content do not induce CYP2B1, CYP3A23, or CYP1A2 gene expression.

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References


Footnotes

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FIGURE LEGENDS
FIG. 1. Effect of *Panax ginseng* extract on rat hepatic CYP2B1, CYP3A23, and CYP1A2 mRNA expression. Adult male rats were administered by oral gavage with a single dose of *Panax ginseng* extract (30 or 100 mg/kg) or an equivalent volume (2 ml/kg) of 0.9% NaCl (vehicle control). As positive controls, another group of rats was injected i.p. with BNF (40 mg/kg once daily for 3 consecutive days), PB (80 mg/kg once daily for 4 consecutive days), or DEX (100 mg/kg once daily for 3 consecutive days). All rats were terminated one day after the last dose and livers were excised. Total RNA was isolated and reverse transcribed. CYP2B1 (panel A), CYP3A23 (panel B), and CYP1A2 (panel C) cDNAs were amplified in duplicates by real-time PCR using gene-specific primers. Data are expressed as the mean ± S.E.M. for four individual rats per treatment group. *Significantly different from the vehicle-treated control group (p < 0.05).*
TABLE 1

*Effect of Panax ginseng and Panax quinquefolius extracts on CYP2B1 and CYP3A23 mRNA expression in primary cultures of rat hepatocytes*

Primary cultures of rat hepatocytes were treated for 48 h with *Panax ginseng* extract (0.1-1000 µg/ml), *Panax quinquefolius* extract (0.1-1000 µg/ml), or culture medium (vehicle control). Cultured hepatocytes were also treated with PB (100 µM; as a positive control for CYP2B1), DEX (10 µM; as a positive control for CYP3A23), or 0.1% DMSO (vehicle control). Total RNA was isolated and reverse transcribed. CYP2B1 and CYP3A23 cDNAs were amplified in duplicates by real-time PCR using gene-specific primers. Data are expressed as the mean (± S.E.M.) fold-increase in mRNA expression (relative to the respective vehicle controls) in hepatocytes isolated from three individual rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CYP2B1 mRNA</th>
<th>CYP3A23 mRNA</th>
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</thead>
<tbody>
<tr>
<td><em>Panax ginseng</em> Extract (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.78 ± 0.05</td>
<td>0.98 ± 0.14</td>
</tr>
<tr>
<td>1</td>
<td>0.86 ± 0.08</td>
<td>0.92 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>0.95 ± 0.23</td>
<td>0.96 ± 0.21</td>
</tr>
<tr>
<td>100</td>
<td>0.93 ± 0.27</td>
<td>0.79 ± 0.01</td>
</tr>
<tr>
<td>1000</td>
<td>0.51 ± 0.25</td>
<td>0.31 ± 0.10*</td>
</tr>
<tr>
<td><em>Panax quinquefolius</em> Extract (µg/ml)</td>
<td>0.84 ± 0.11</td>
<td>0.78 ± 0.01</td>
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<tr>
<td>1</td>
<td>0.78 ± 0.07</td>
<td>0.60 ± 0.08</td>
</tr>
<tr>
<td>10</td>
<td>0.88 ± 0.14</td>
<td>0.72 ± 0.06</td>
</tr>
<tr>
<td>100</td>
<td>1.16 ± 0.26</td>
<td>1.00 ± 0.20</td>
</tr>
<tr>
<td>1000</td>
<td>0.47 ± 0.11*</td>
<td>0.87 ± 0.14</td>
</tr>
<tr>
<td>PB (100 µM)</td>
<td>49 ± 22*</td>
<td>not determined</td>
</tr>
<tr>
<td>DEX (10 µM)</td>
<td>not determined</td>
<td>41 ± 2*</td>
</tr>
</tbody>
</table>

*Significantly different from the corresponding vehicle-treated control group (p < 0.05).**
Fig. 1

(A) CYP2B1

(B) CYP3A23

(C) CYP1A2